

SECONDARY METABOLISM IN *PENICILLIUM CYCLOPIUM*

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ABSTRACT

Dimethylallyl transferase (E.C.2.5.1.1.), isopentenylpyrophosphate isomerase (E.C.5.3.3.2.) and a prenyl-aryl transferase, enzymes involved in the production of a cyclopiazonic acid in *Penicillium cyclopius* Westling, were isolated, and the isoelectric points measured. These were for dimethylallyl transferase 5.2, isopentenylpyrophosphate isomerase 4.9 and prenyl-aryl transferase 5.6. *P.cyclopius* was grown on NaNO_3 -containing medium, where nitrogen was limiting, and showed low volume mycelial production and high α and β cyclopiazonic acid production. *P.cyclopius* was then grown on corn steep liquor and showed high mycelial production and almost no α and β cyclopiazonic acid production, and yet it was found by experimentation that the prenyl-aryl transferase was present and active in *P.cyclopius* grown on corn steep liquor. The ability of *P.cyclopius* when grown on corn steep liquor to produce the prenyl-aryl transferase means that much higher quantities of the prenyl-aryl transferase can now be isolated, as *P.cyclopius* grown on corn steep liquor has a larger volume of enzyme-containing mycelia. A novel method was found for isolating α -acetyl γ (β indolyl) methyl tetramic acid and trp- γ and α (unknown intermediates in cyclopiazonic acid production) from large quantities of α and β cyclopiazonic acid, by use of a Sephadex G10 column and an elution buffer of 1mM EDTA adjusted to pH 10.5 with ammonia. The effect of 2-(p-chlorophenoxy)-2 methylpropionic acid (Clofibrate), an inhibitor of hydroxymethylglutaryl coenzyme A reductase in mammals, was studied in *P.cyclopius* in an attempt to inhibit hydroxymethylglutaryl coenzyme A reductase activity. It was hoped that inhibition of isopentenylpyrophosphate and dimethylallylpyrophosphate production would lead to an increase in the levels of α -acetyl γ (β indolyl) methyl tetramic acid. However this did not occur and cyclopiazonic acid was still produced, suggesting that Clofibrate does not inhibit hydroxymethylglutaryl coenzyme A reductase in *P.cyclopius*.

DECLARATION

I declare that this dissertation is my own unsided work.
It is being submitted for the degree of Master of Science
in the University of the Witwatersrand, Johannesburg. It
has not been submitted before for any degree or examina-
tion in any other University.


Barrington Herman

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List of Abbreviations

cAAtrp	cyclo-acetoacetyl-L-tryptophanyl
CA	cyclopiazonic acid
Clofibrate	2-(p-chlorophenoxy)-2methylpropionic acid
CPIB	chlorophenoxyisobutyric acid
CSL	corn steep liquor
DEAE	diethylaminoethyl
DMAPP	dimethylallyl pyrophosphate
DTE	2,2'-dihydroxy-1,4-dithiolbutane
HMG-CoA reductase	β -hydroxy- β -methylglutaryl Coenzyme A reductase
IPP	isopentenylpyrophosphate
I	isopentenylpyrophosphate isomerase
PAGE	polyacrylamide gel electrophoresis
PPO	2,5-diphenyloxazole
S	prenyl-aryl transferase
T	dimethylallylpyrophosphate transferase
TEMED	N,N,N',N'-tetramethylethylenediamine
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol

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CHAPTER 1

INTRODUCTION

PRIMARY AND SECONDARY METABOLISM

In all living organisms biological components are synthesised and utilised, these processes are all grouped under the heading of metabolism, which is broken into two categories, anabolism (synthesis) and catabolism (degradation). All organisms possess similar pathways by which they synthesise and utilise certain essential biological compounds such as sugars, amino acids, fatty acids, nucleic acids and the polymers associated with these basic building blocks. These compounds are all essential to the well being and survival of the organism and are termed primary metabolites and are synthesised by primary metabolism.

Many organisms also produce products which have no apparent utility and these are termed the natural products or the secondary metabolites, and their pathways of synthesis and utilisation are the pathways of secondary metabolism.

The division between primary and secondary metabolism is often hazy, as there are obscure amino acids which may be regarded as secondary metabolites, while many of the sterols have essential roles and must be regarded as primary metabolites. The situation is further complicated as the precursors for secondary metabolism are provided at branch points in primary metabolism, for instance the primary amino acids lead to the formation of alkaloids and peptide antibiotics.

The purpose for which organisms produce secondary metabolites is not completely understood, but many hypotheses have been advanced to explain them. In 1947 Foster suggested that secondary metabolites were formed, because under conditions of carbohydrate excess, saturation of the enzymes involved in complete oxidation of the substrate leads to the accumulation of intermediates which are non-essential and are thus excreted or shunted to subsidiary enzyme cycles. Bu'Lock (1965) proposed the terms trophophase and idiophase to differentiate the two broad phases shown to occur in the development of submerged cultures. In the trophophase balanced

replication of cell material and uptake of nutrient occurs. This phase is usually terminated by a number of factors these usually being the depletion of an essential nutrient. This is followed by the idiophase during which physiological changes occur and secondary metabolites are produced. It has been suggested by various authors (Woodruff 1966; Bu'Lock 1975; Berry 1975) that on exhaustion of a macronutrient primary metabolism becomes unbalanced and this leads to the accumulation of various intermediates. This balance can be regained by removing these intermediates and thus allowing the organism to survive. The intermediates are removed from the system by building essentially "useless" molecules. In the event of the limiting nutrient being supplied, these "useless" molecules can then be catabolised to supply both building blocks and energy.

An alternative hypothesis is that the secondary metabolites are produced for ecological reasons. Many of the secondary metabolites produced by plants are highly toxic and could be a defensive mechanism, and many insects use secondary metabolites in their everyday life, such as pheromones (Mann 1979). The above hypothesis is to some extent supported by Luckner (1972), although he points out that, considering the great number of secondary metabolites formed, the number used for these purposes are small and their biological significance seems to be a random process which evolved after its occurrence rather than being a reason for their selection.

Throughout history man has used these natural products, usually in the form of crude plant extracts, for relief from pain and the alleviation of the symptoms of disease, examples being morphine from two species of poppy, ricinine a purgative (castor oil). Salicin from willow bark was used for fevers and is now produced synthetically as aspirin. Poisons such as curare were used for hunting and warfare and conine from hemlock is a well known poison. The Indians of South America have been using the pulp of the Cocao leaves (cocsine) and caffeine as stimulants for centuries. Throughout the ages man has also used odorous compounds such as rose oil

and oil of lavender to mask his natural body odours and spices such as cinnamon, cloves and garlic as seasoning to hide the taste of bad food.

It is not only plants that have been known to produce secondary metabolites, many fungi are known to produce them, along with insects and animals. Between 1815 and 1860 more than 20 natural products were isolated, including morphine, quinine, cocaine, caffeine, nicotine, codeine and camphor, although their chemical synthesis took a little longer, eg. morphine in 1952.

In 1882 Huseman, Hilger & Huseman discussed the isolation of a toxic alkaloid substance from mouldy bread. Wronin in 1891 published an article on the toxic properties of *Gibberella zeae* infected rye. Mitchell in 1918 described experiments in which he was able to induce symptoms of poisoning in cattle similar to those observed in field cases, by feeding maize artificially infected with *Diplodia zeae*. Not all secondary metabolites produced by the fungi are toxins, penicillin and streptomycin, for example, are potent antibiotics used in medicine.

For the three decades after the publication by Mitchell in 1918, there was very little study of the fungal natural products. Then in 1945, Drobotko observed that the large number of horse mortalities in the USSR were caused by Stachybotryotoxicosis which was shown to follow the ingestion of fodder contaminated by *Stachybotryx atra*.

Following this the role of fungi in a number of veterinary toxicoses of unknown aetiology was investigated. *Pithomyces chartarum* was established as the causative agent in the "facial eczema syndrome" which had been responsible for severe losses among cattle in Australia and New Zealand (Thorton & Ross 1959; Thorton & Percival 1959). During the same decade Forges and Carll implicated strains of *Aspergillus flavus* and *Penicillium rubrum* in outbreaks of "mouldy corn toxicosis" which had affected large numbers of swine in the USA during the period 1952-1959, and they were also successful in reproducing the symptoms of bovine

hyperkeratosis in experimental animals using strains of *Aspergillus clavatus* and *Aspergillus chevalieri* isolated from feed implicated in field cases of the disease (Burnside *et al* 1957; Forgacs & Caril 1962).

The mortality of a large number of turkey poults, ducklings, swine and cattle were shown to have, as causative agents, the aflatoxins, a group of structurally related metabolites of *Aspergillus flavus*. They proved not only to be extremely toxic but also to possess definite hepatocarcinogenic properties. The most toxic of these metabolites aflatoxin B₁ has LD₅₀ values which range from 10mg/kg for month old hamsters to 0.5mg/kg for dogs, trout and day old ducklings (Wogan 1966). Tumours were induced in a number of experimental animals following oral doses of aflatoxin for prolonged periods at sublethal levels (Lancaster *et al* 1961; Barnes & Butler 1964; Newberne 1965). As *Aspergillus flavus* has the ability to grow on a wide variety of crops and to produce aflatoxins on them all, further study is essential as these toxins have been found in processed foodstuffs ready for human consumption.

Japanese workers, working with mycoflora causing the yellowing of rice, discovered two metabolites of *Penicillium islandicum* which are acutely toxic in nature. Luteoskryn, one of these metabolites, has been shown, after long term administration to experimental animals, to lead to the formation of hepatomas (Miyake *et al* 1959; Kobayaski *et al* 1959; Miyake *et al* 1960; Uraguchi *et al* 1961). It has been suggested by Kraybill and Shimkin (1964) that as the aflatoxins and *Penicillium islandicum* toxin have a hepatocarcinogenic nature that there might be some correlation between the high occurrence of hepatic carcinomas amongst African and Oriental peoples and the fungal contamination of maize and rice, the basic components of the majority of these peoples diets.

In 1965 Scott carried out an investigation in which he tested 228 mould strains for toxicity. To do this he isolated the mould from cereal and legume crops and grew it for two weeks on maize meal cultures. After this the cultures were dried and crushed and then included in the diets of the

test animals (Peking ducklings) for two weeks. Forty six strains representing 22 of the 59 species examined were found to cause the death of all the animals in the test period. A single strain of each of the 22 species was then tested for toxicity in newly weaned rats and mice. The feeding trials were carried out over a four week period. Meals that had been infected with *Aspergillus avenaceus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Penicillium oxalicum* and *Penicillium urticae* were shown to produce toxic agents effective against both rats and mice. Scott later re-identified *Penicillium urticae* as *Penicillium cyclopium* and the toxin from this brought about the death of all animals in the test group within one week.

Holzappel (1968) cultivated *Penicillium cyclopium* on maize meal and extracted the toxin quantitatively using chloroform-methanol, and then isolated cyclopiazonic acid, a novelly substituted tetramic acid, by chromatography on formamide-impregnated cellulose and ion-exchange columns. See Figure 1 for the structure of Cyclopiazonic acid.

Cyclopiazonic acid is an optically active colourless crystalline compound with the empirical formula $C_{20}H_{20}N_2O_3$. The compound titrates as a monobasic acid and forms the monohydrazone $C_{20}H_{22}N_4O_2$ and gives the intense orange/red ferric colour reaction characteristic of the intermolecularly H_2 bonded enolized β diketones, and the purple/blue Ehrlich colour of an indole unsubstituted at either the α or β position.

The mass spectrum of the toxin shows a strong molecular ion at m/e 336 and prominent fragmentations at m/e 154, 155, 181, 182 and 196. The U.V. spectrum of the toxin in methanol exhibits absorption maxima at wavelengths 225, 253, 275(sh), 284 and 292 nm. The log molar extinction coefficients are 4.60, 4.22, 4.28, 4.13 and 4.24 respectively. The toxicity of cyclopiazonic acid was examined and the LD_{50} for day old ducklings was found to be 2.4mg/kg body weight (Neethling 1972). Purchase (1974) found that the toxin causes changes in the kidney, heart, pancreas and spleen.

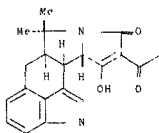
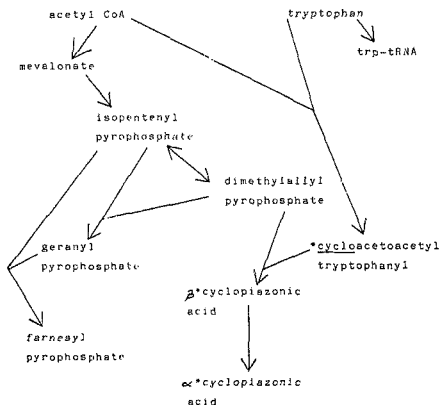


Figure 1. Structure of Cyclopiazonic acid

Holzappel & Wilkins (1971) found that labelled tryptophan(24.7%), mevalonate(7.3%) and acetate(3.5%) were incorporated into α -CA and by using chemical degradation showed the positional incorporation of labelled mevalonate and acetate. In 1970, Holzappel *et al* isolated two new metabolites, and in 1971 Holzappel and Wilkins showed that one of these, β -CA was an intermediate during the biosynthesis of α -CA. In 1971 Schabert In 1971 Schabert *et al* extracted five isoenzymes of cyclopiazonate oxidocyclase. These were responsible for converting the intermediate β -CA to α -CA. Studies on the mechanisms, kinetics, inhibitors, amino acid composition and flavin prosthetic groups of the five isoenzymes were carried out by Schabert and Potgieter (1971) and Schabert and Steenkamp (1976).

H^C Grath *et al* (1973) isolated an intermediate in α -CA formation. It was identified as *cyclo*-acetoacetyl-tryptophanyl (α acetyl- γ -(β -indoyl) methyl tetramic acid) and in 1976 they found that it was maximally produced on the fourth day of growth. In 1976-1977 H^C Grath *et al* isolated the enzyme dimethylallylpyrophosphate:*cyclo*-acetoacetyl-tryptophanyl dimethylallyl transferase (secondary transferase) which converts cAAtrp into CA, and demonstrated that dimethylallylpyrophosphate (DMAPP) and cAAtrp were the substrates. They found that the enzyme was specific for these substances and that cAAtrp production was initially rate limiting. When this substance pooled however the production of DMAPP was rate limiting. The regulatory mechanism of the secondary transferase is not known. Figure 2 shows the positions of the primary and secondary metabolic routes under investigation by H^C Grath and Herman.

Penicillium cyclopium has also been shown to produce, in addition to cyclopiazonic acid, the phenylalanine and anthranilic acid derived quinoline alkaloids, viridicatin and viridicatol (the pathway for the production of viridicatin is shown in Figure 3) (Cunningham & Freeman 1953; Bracken *et al* 1954; Birkenshaw *et al* 1963). It must be remembered that the production of these benzodiazepine alkaloids is not the same as that of cyclopiazonic acid production. Viridicatin and viridicatol are



The asterix marks the secondary metabolites

Figure 2. Cyclopiazonic acid Synthesis: The relationship of CA synthesis to primary metabolism

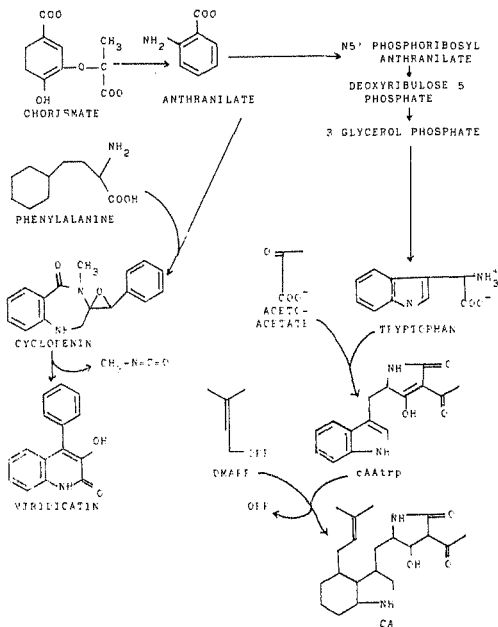


Figure 1 Secondary Metabolism of *I. niger* - Some of the metabolites, and their pathways of formation, in *I. niger*.

only produced during maturation of the conidiospores, and in *P. cyclopium* the enzyme cycloprenase is located on the inside of the plasma membrane, inaccessible to its substrates. *Penicillium cyclopium* has also been shown to produce 6-methylsalicylic acid which contains no nitrogen, against CA containing two molecules of nitrogen. When nitrogen is limiting α -CA is produced, and if nitrogen and carbon concentrations are raised, 6-methylsalicylic acid is produced (M^c Grath and Hartley unpublished observations).

When nitrogen levels are reduced in the medium, there is after approximately 48 hours a general drop in *P. cyclopium* metabolism, CO₂ evolution, the rate of increase of biomass, and protein production all decrease (Neethling and M^c Grath 1977). Because of this drop in metabolism acetyl coenzyme A (AcCoA) an important energy source and one of the controllers of the tricarboxylic acid cycle must be either diverted or pooled. Tryptophan, whose synthesis continues at a decreasing rate, is removed along with two molecules of AcCoA as cAATrp. The secondary transferase joins cAATrp to DMAPP (formed from 3 molecules of AcCoA) to form β -CA. Competition for DMAPP between the primary and secondary transferases is thought to be controlled by the appearance of cAATrp (Neethling and M^c Grath 1977). Neethling and M^c Grath (1977) point out that this agrees with Bu'Lock's (1975) suggestion that secondary metabolism links various important metabolites or processes in primary metabolism.

M^c Grath *et al* 1976 & 1977 purified the two enzymes, primary transferase and secondary transferase, as it was thought that the competition between the two enzymes may be the rate controlling step. They found that both enzymes were produced at the same time and that maximal production of the secondary transferase preceded maximal CA production by at least 24 hours. The amount of secondary transferase was well in excess of that needed to produce the amounts of CA found. They also found that a divalent cation chelator such as EDTA inhibited the primary transferase, which requires Mg²⁺, but not the secondary transferase.

α -CA is also a divalent cation chelator (see figure 4) (Steyn & Rabie 1976; Dippenar *et al* 1978). Mc Grath *et al* (1976) found that it inhibits the primary transferase, and has a feedback inhibition effect on the secondary transferase. Thus once α -CA is produced, the pathway through the secondary transferase would be favoured. The primary transferase has a sharp pH optimum at 7.4 whilst that of the secondary transferase is broad at around pH 6-8. They both possess a subunit structure which appears to be variable dependant on the different growth of the organism. Mc Grath *et al* (1976 & 1977) suggested that the pH may be another controlling factor, as the primary transferase activity is lowered with the drop in pH, whilst the secondary transferase is relatively unaffected.

The secondary and primary transferases do much the same job, in that both remove the pyrophosphate from dimethylallylpyrophosphate and then join the dimethylallyl moiety to another molecule. The primary transferase to isopentenylpyrophosphate and the secondary transferase to *cyclo*-acetoacetyl-tryptophanyl.

The main aim of this project was to extract the isopentenylpyrophosphate isomerase, dimethylallylpyrophosphate transferase (primary transferase) and the prenyl-aryl transferase (secondary transferase) of *Penicillium cyclopium* in a stable form, and using various techniques to attempt their partial or full purification. The isomerase and primary transferase of pig liver were extracted (there is no secondary transferase in pig liver) for use in mixed enzyme assays with the enzymes of *P. cyclopium* as the pig liver enzymes are fairly easy to isolate, and large amounts of isomerase were needed to assay for the primary transferase of *P. cyclopium*. *Penicillium cyclopium* grown on corn steep liquor produces large amounts of mycelium (but little CA). Therefore the question arises as to whether or not the prenyl aryl transferase (S) would be present in such a culture. If it were present in large quantities then it would be the preferred method for producing large amounts of the enzyme. This would also have implications concerning the control of the secondary metabolite production or enzyme expression.

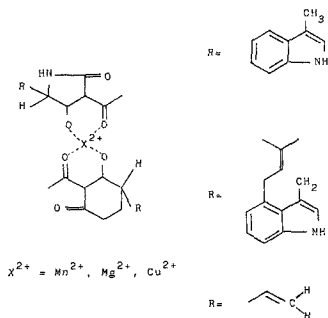


Figure 4. Metal Chelation by Tetramic Acids

In order to assay for the secondary transferase, the isolation of one of its substrates *cyclo*-acetoacetyl-L-tryptophanyl (the other is dimethylallylpyrophosphate which was synthesised from isopentenylpyrophosphate using the ~~isomerase~~) ~~was essential and eventually~~ formed a great part of this work. Attempts at increasing the yields of *cyclo*-acetoacetyl-L-tryptophanyl were to be made, based on the principle that inhibition of the enzyme β -hydroxy- β -methylglutaryl Coenzyme A reductase using Clofibrate (a drug used in the suppression of cholesterol synthesis) should theoretically lead to an increase in the amount of *cyclo*-acetoacetyl-L-tryptophanyl.

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture Medium

(A) Minimal medium

The medium was adapted from the minimal medium previously described by Neethling & M^c Grath (1976). One litre of the medium consists of the following macronutrients in g:

Glucose	60
NaNO ₃	4.5
K ₂ HPO ₄	0.121
KH ₂ PO ₄	0.87%
MgSO ₄	0.5
KCl	0.5

and the following micronutrients in mg:

Na ₂ B ₄ O ₇ ·H ₂ O	7
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	5
CuSO ₄ ·5H ₂ O	3
MnSO ₄ ·4H ₂ O	1.1
ZnSO ₄ ·7H ₂ O	176
FeSO ₄ ·7H ₂ O	100

The pH was adjusted to 5.8 - 5.9 using HCl, then 100ml aliquots of the medium were measured into 500 ml Erlenmeyer flasks and loosely sealed with aluminium foil. These were then sterilised at 15 lbs, 121°C for 15 min. It was found that the flasks, containing medium, could be autoclaved without caramelization or precipitation of the nutrients or alteration of the pH, if the steam pressure was released rapidly, with care being taken that the medium did not boil.

(B) CSL medium

CSL was used in some experiments as an alternative nitrogen source to NaNO₃. The minimal medium was made up as in (A) above, but the NaNO₃ was

replaced by 3% (w/v) CSL. The CSL medium was then treated in the same way as the minimal medium in (A) above.

2.2a Storage of *Penicillium cyclopium*

Spores of *Penicillium cyclopium* Westling CSIR 1082 were stored in liquid nitrogen for use as starting material for the inoculum. Cultures were removed from liquid nitrogen and allowed to thaw at room temperature. Spores were aseptically transferred from these cultures to malt agar plates and incubated at 25°C for seven days. After seven days spores were collected from the malt agar plates and aseptically transferred to minimal medium agar plates and incubated at 25°C for seven to fourteen days. Minimal medium agar is made from minimal medium (2.1) with 1% agar added.

Stock cultures were prepared using minimal medium with the agar omitted. The solution was autoclaved as above and once cool was aerated on a shaker for approximately one hour. Aseptically 2 ml of this medium was pipetted into vials and the vials inoculated with spores from the minimal medium agar plates. The vials were incubated at 25°C in a well humidified room. After approximately 10 days the surface of the medium was well covered with mycelial mat and spores. The mat and spores were well washed, by decantation with sterile distilled water, before being dried under vacuum, and stored at either 5°C, -70°C or -180°C.

2.2b Preparation of the Inoculum, and Growth, of *Penicillium cyclopium*

Inoculation: The dried mycelial mat (2.2a) was flooded with approximately 5ml of sterile medium, using aseptic techniques. The medium now containing the slant was added back to the culture medium in the Erlenmeyer flask and homogenised using an Ultra Turrex. The flask containing the slant was then placed on a rotary shaker (New Brunswick model G10 gyratory 2" orbit) at 180 rpm and 25°C.

replaced by 3% (w/v) CSL. The CSL medium was then treated in the same way as the minimal medium in (A) above.

2.2a Storage of *Penicillium cyclopium*

Spores of *Penicillium cyclopium* Westling CSIR 1082 were stored in liquid nitrogen for use as starting material for the inoculum. Cultures were removed from liquid nitrogen and allowed to thaw at room temperature. Spores were aseptically transferred from these cultures to malt agar plates and incubated at 25°C for seven days. After seven days spores were collected from the malt agar plates and aseptically transferred to minimal medium agar plates and incubated at 25°C for seven to fourteen days. Minimal medium agar is made from minimal medium (2.1) with 1% agar added.

Stock cultures were prepared using minimal medium with the agar omitted. The solution was autoclaved as above and once cool was aerated on a shaker for approximately one hour. Aseptically 2 ml of this medium was pipetted into vials and the vials inoculated with spores from the minimal medium agar plates. The vials were incubated at 25°C in a well humidified room. After approximately 10 days the surface of the medium was well covered with mycelial mat and spores. The mat and spores were well washed, by decantation with sterile distilled water, before being dried under vacuum, and stored at either 5°C, -70°C or -180°C.

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Growth: The above culture was allowed to grow for approximately 72 hours and was used as the primary inoculum. This primary inoculum was then homogenised with an Ultra Turrex and 5ml aliquots of the homogenate were used to sub-inoculate 100ml batches of fresh medium. These flasks were then shaken at 180 rpm at 25°C for various times depending on the experiment.

2.3a Extraction of Metabolites of *Penicillium cyclopium*

CA and intermediates of its biosynthesis were isolated from cultures of *P. cyclopium* using two methods. Method 1 was used primarily to obtain cAATrp and α - and β -CA whilst method 2 as well as extracting the above also extracted the more polar trp-x and-y. In both procedures the mycelium and medium were separated by filtration.

Method 1

Filtrate: Approximately 80ml of filtrate was obtained from each flask, to which was added: 50ml of chloroform and 2ml 2M HCl. This was then shaken at 180 rpm for 30 min, the aqueous and organic layers were then separated by centrifugation at 2000 rpm for ten min and the chloroform layer removed. The chloroform layer was washed with two 20ml portions of 0.75M ammonia and in each case the aqueous layer retained. These aqueous layers were acidified and washed with two 10ml portions of chloroform. The chloroform containing the metabolites was then taken to dryness using a rotary evaporator.

Mycelium: The mycelia were stirred into 50ml of chloroform and 0.5ml concentrated HCl and were then macerated using an Ultra Turrex and the homogenate shaken for 30 min. The chloroform was then aspirated off and treated in the manner described above.

Method 2

Filtrate: For each flask the filtrate was taken down to dryness using a rotary evaporator and the residue resuspended in 10ml of methanol. The methanol insoluble material was removed by filtration.

Mycelium: 80ml of methanol was added to the mycelium which was then homogenised, this homogenate was then shaken at 180 rpm for 30 min, and the methanol was filtered off.

2.3b Separation of Metabolites of *Penicillium cyclopium*

A: Thin Layer Chromatography (t.l.c.)

Merck F₂₅₄ fluorescent silica gel thin layer plates (0.25 mm thick) were used to separate the metabolites. The metabolites were either streaked or spotted onto the plate which was then run in an ethylacetate/methanol/ammonia-25% (20:5:3 by vol) system. Identification of the metabolites on the plate was done by a combination of two methods.

1; u.v. light: the plates were scanned under 254nm light and bands of absorbance marked

2; Ehrlich spray: (Stahl 1965) 1g 4-dimethylamino benzaldehyde was dissolved in 25ml concentrated HCl to which was added 75ml of 96% ethanol.

The Ehrlich spray interacts with the indole rings causing them to be destroyed and the metabolites cannot be recovered to assay. However with u.v. light the bands can be scraped off and measured quantitatively in a spectrophotometer. As the u.v. light highlights any molecule absorbing at 254nm this method must be used in conjunction with Ehrlich spray in order to pick up only the molecules of interest.

The various metabolites, after separation, were extracted from the silica gel with methanol, and the concentration was determined spectrophotometrically by reading at 280nm using a methanol blank. The

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The various metabolites, after separation, were extracted from the silica gel with methanol, and the concentration was determined spectrophotometrically by reading at 280nm using a methanol blank. The

log molar extinction coefficient is 4.31, and the concentration calculated from this.

B: Column Chromatography

Sample: The metabolites were extracted as previously mentioned. After being taken to dryness on the rotary evaporator the extract was resuspended in 60ml chloroform. This was then dispensed in 1ml aliquots into 60 vials, taken to dryness in a desiccator and stored in a -20°C freezer. The vials were removed as needed and the dried extract resuspended in 5ml chloroform.

Column Packings and Techniques.

Various column packings and techniques were used in an attempt to separate cAATrp from the other secondary metabolites of *P.cyclopium* :

1. XAD-2: Amberlite XAD-2 (Amberlite registered trademark of Rohm and Haas supplied by Sigma) is a non ionic polymeric adsorbant (see discussion) and is a copolymer of styrene and divinylbenzene. The XAD-2 was washed in distilled water and the fines removed. The XAD-2 was suspended in distilled water and then poured into a Wright column (30 by 15cm). The column was equilibrated using distilled water pumped by an LKB varioprep pump at 1ml/min. The sample was added to the column via the pump and fractions of various sizes, depending on the experiment, collected. The column was eluted using various concentrations of methanol (see figures 12, 13 and 14 in the Results section). At the end of each run the column was washed with 50ml of 100% methanol followed by 50ml of propanol and finally 50ml of distilled water.
2. Silica gel: Silica gel is a polar adsorbant and was used primarily because of the excellent separation of the metabolites on thin layer plates. A Wright column (30 by 15 cm) was packed using Merck silica

gel, Kieselgel 60, 70 - 230 mesh. The column was equilibrated using ethylacetate/ammonia/methanol at 95:2.5:2.5 (v/v/v). The column was eluted isocratically at either 1ml/min or 0.5ml/min. (See figure 16 in the results section) Sephadex G10 : Sephadex G10 is a low water regain dextran gel and was used not only for its sieving effects, but also for its ionic and aromatic interactions (see Discussion). A Wright column (30 by 15 cm) was packed with Sephadex G10. The Sephadex was allowed to swell and then degassed and poured into the column using a glass rod to prevent aeration. The column was equilibrated for 24 hours using distilled water, pumped at 1ml/min using an LKB varioperpex pump. The sample was added to the column via the pump.

Various buffers were used to elute the sample, and their constitution can be seen in the related figures (fig 17 - 23) in the Results section.

2.4 Enzyme Assays for *Penicillium cyclopium* and Pig Liver.

One unit of enzyme activity is defined as that amount which causes the transformation of 1.0 μ mol of substrate per minute at 25°C. The Specific Activity is defined as the number of enzyme units per mg of protein.

Enzyme assays were carried out using [1-¹⁴C] isopentenylpyrophosphate, 200 μ l containing 10 microcurie, supplied by Amersham U.K. The incubation temperatures were 25°C; 30°C or 37°C or as shown in the results.

The incubation medium for both the isomerases (i.e. *P.cyclopium* and pig liver) was: 10mM TRIS HCl pH 6; 5 μ moles MnSO_4 ; 59.43n moles [1-¹⁴C] IPP, 110 000 dpm in one ml.

The incubation medium for both the transferases (i.e. *P. cyclopius* and pig liver) was: 10mM TRIS HCl pH 7.6; 5 μ moles $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 59.43n moles [$1\text{-}^{14}\text{C}$] IPP, 110 000 dpm 50 μ l purified isomerase in one ml.

The incubation medium for the secondary transferase was: 10mM TRIS HCl pH 6.4; 5 μ moles MnSO_4 ; 59.43n moles [$1\text{-}^{14}\text{C}$] IPP 110 000 dpm; 50 μ l purified isomerase; 7.5 μ g purified cAATrp in one ml.

The assay mediums were incubated for 5 min. once the sample was added, or in the case of the transferase assay, once the isomerase was added, as the transferase could not be assayed for without the isomerase. (see figure 5).

On completion of the incubation period, the reaction was stopped by the addition of a few drops of concentrated HCl saturated with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Toluene (1ml) containing geraniol, nerolidol and farnesol each at 1% v/v to toluene, was added to each assay tube to extract the product. This mixture was agitated briefly. The solutions were then centrifuged at 2000 rpm. for 5 min to break the suspension. A 0.5ml sample of the toluene layer in each tube was taken and added to 4.5ml aliquots of toluene containing 0.4% PPO. Radioactivity was measured in a Beckman LS 250 scintillation counter. The secondary transferase was assayed in the same manner as described above, except that the 0.5ml toluene layer sample was streaked onto a t.l.c. plate and developed as described (2.3b). The silica gel bands containing α - and β -CA were then scraped off and the radioactivity counted.

2.5.1 Enzyme Purification for *Penicillium cyclopius* and Pig Liver

Extraction

The media for extracting enzymes from both *P. cyclopius* and pig liver were similar, containing the following in 1L of distilled water:

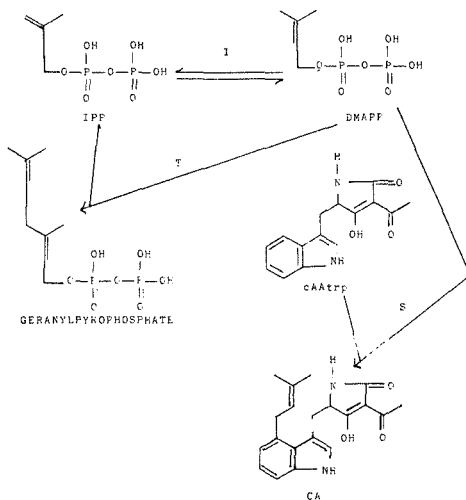


Figure 5. Mode of action of the transferase, isomerase and secondary transferase

TRIS HCl 10mM

DTE 1mM

EDTA 1mM

The TRIS HCl pH for *P.cyclopium* was 7.2 and for pig liver was 7.6

For *P.cyclopium* mycelia were separated from the culture media by filtration. Mycelial mats were removed from the Buchner funnel after filtration and immersed in an acetone/dry ice solution and allowed to thaw up to room temperature (approx. 120 min). The mycelia were then separated from the acetone/dry ice solution by filtration and allowed to dry. The dry mycelia were then crushed using a mortar and pestle and the resulting powder stored at -20 C until needed. Other methods of mycelial disruption used were the Waring blender and the Potter-Elvehjem homogeniser. However using the same extraction media these were found not to be suitable (see Results).

For pig liver, the livers of freshly slaughtered pigs were supplied as gifts by the Krugersdorp abattoir, 300g of the liver was diced into small cubes (approx 2cm by 2cm) and homogenised in a Waring blender in cold extraction medium.

2.5.2 Centrifugation

For pig liver the homogenate was centrifuged at 16300g for 20 min at 5°C, the supernatant was then aspirated off and centrifuged again at 48 200g for 3 hours at 5°C. Excess fat was removed by filtration through cotton wool. For *P.cyclopium* the acetone/dry powder was reconstituted in extraction medium and centrifuged as above.

Thereafter the *P.cyclopium* and pig liver extracts were treated similarly, as described in the following sections.

2.5.3. Ammonium Sulphate Fractionated Samples

The crude enzymes (from 2.5.1) were fractionated with ammonium sulphate in the concentration ranges 0-30%; 30-40%; 40-50%; 50-60% and finally 60-70%. The precipitated protein being centrifuged down in each fractionation range at 48000g for 15 min at 2°C. The precipitate was re-suspended in the extraction medium and then dialysed against extraction medium for 16 hours (2 medium changes).

2.5.4 Non Ammonium Sulphate Fractionated Samples

The final supernatant from either *P.cyclopium* or pig liver was not treated with ammonium sulphate but was instead dialysed against solid sucrose for 4-5 hours. After this the dialysis sac was tightened and the supernatant dialysed against extraction medium for 16 hours (2 changes) and the concentrated diffusate was then stored in 5ml aliquots at -20°C.

2.5.5 Gel Chromatography of the Enzymes of *Penicillium cyclopium* and Pig Liver

The gel filtration chromatography was carried out using a 120 by 2cm column, packed with Ultrogel AcA 34 having a linear fractionation range of 20 000 to 350 000 and an exclusion limit of 750 000. The column was run under hydrostatic pressure of 35 cm.

Method 1: The protein sample was taken from the previous ammonium sulphate fractionations, each of the various fractions being adjusted to 7mg/ml protein for purposes of comparison. These fractions, 30-40%; 40-50%; 50-60% and 60-70% were separately dialysed and each fraction was loaded and eluted from the column before the next fraction was added. Holoway and Popjak (1966) showed, for pig liver only, that the primary transferase precipitates in fraction 40-50% and the isomerase in fraction 60-70%, hence the separate loading and running of the fractions. The isomerase fraction was run first because it is easily assayed and the transferase cannot be assayed for without it.

Method 2: The whole sample i.e. the sucrose concentrated, dialysed unfractionated, centrifuged sample, was adjusted to have a concentration of 7mg/ml of protein and then a sample of 3-6% of the column volume was added to the column. It was found that complete separation of the isomerase and transferase could be obtained. In this way a large amount of enzyme was built up without recourse to ammonium sulphate fractionation.

2.5.6 Ion Exchange Chromatography of the Enzymes of *Penicillium cyclopium* and Pig Liver

A 30 by 0.8cm column packed with Whatman DE 11 was used for these separations. The DE 11 was precycled in order to improve the reproducibility of the results.

For the precycling treatment the ion exchanger was stirred into 15 volumes of 0.5N HCl and left for 30 min, the supernatant was filtered off until the eluate was at pH 4. The ion exchanger was then stirred into 15 volumes of 0.5N NaOH and left for 30 min, this was then washed in a funnel until the eluate was near neutral.

The fines were then removed using the formula $t = nh$ where t = time (min) allowed for settling, h = the total height of the measuring cylinder(cm) and n is a factor between 1.3 and 2.4, the factor of 1.3 removes all fines, but can lead to loss of material, whereas the factor of 2.4 leads to removal of only the finest particles. An n factor of 1.8 was used in the fines removals in all of these experiments. The DEAE was then degassed and packed into the column, using a glass rod so as not to aerate the slurry.

The column was equilibrated by running buffer at 5 times the concentration but the same pH as the starting buffer through the column, and then passing starting buffer through the column for 24 hours.

2.5.7 Isoelectric Focusing of the Enzymes of *Penicillium cyclopium*

Originally it was intended to fabricate apparatus along the lines of Valmet's zone convection electrofocusing apparatus (Valmet 1969), however this apparatus could not be made watertight. Equipment was then fabricated for use with a BioRad electrofocusing plate.

50g of Bio Lyte electrofocusing gel was swelled in 1800ml of distilled water for 4 hours. The hydrated gel was then washed with 10 litres of distilled water, on Whatman 41 filter paper in a large Buchner funnel. After washing the gel was transferred to a 2L glass beaker using the minimum amount of distilled water. The gel was allowed to settle overnight and excess water was then aspirated off. The gel was then stored at 4°C, 5ml of chloroform being added to act as a preservative.

200ml of the gel was mixed with various ampholines to give solutions of 3%. Electrofocussing wicks were cut to size, placed at the ends of the plate and wet with distilled water. 150ml of the gel/ampholine/sample solution was then poured into the plate. Filter paper squares were placed on the electrofocussing wicks and the excess moisture drawn off. The apparatus was then run at 500 volts 15 milliamps (VOKAM 500/150 power pack) or 8 watts (BioRad power pack) overnight.

Sample: The sample was prepared by taking the enzyme solution (reconstituted powder see 2.5.1) and dialysing against 10mM TRIS HCl pH 7.2, 1mM DTE for 16 hours. The non diffusable material was then adjusted to 50mg/ml and 4ml aliquots of this were then lyophilised. The lyophilised sample was added to the gel, prior to pouring the bed.

Harvesting: After running, the bed was checked visually for bands and then a 30cm strip of fluorescent silica gel on a mylar backing was inserted edgewise into the gel. Once fluid had risen to within 3mm of the top of the strip, it was removed and the protein bands checked by illumination with u.v. light at 254nm.

2.5.8 Polyacrylamide Gel Electrophoresis of the Enzymes of *Penicillium cyclopium* and pig liver

Vertical slab gel electrophoresis was carried out using a continuous buffer system, the separating gel consisted of 8.3ml of 30% w/v acrylamide, 6.7ml of 1% w/v bisacrylamide, 6.2ml of 1.5M TRIS (HCl) at pH 8.7 and 3.4ml of distilled water, this was mixed, degassed, and had 10 μ l of TEMED and 100 μ l of 10% w/v ammonium persulphate added.

The glass plates were cleaned with ethanol, and the spacers were smeared with vaseline. The glass plates were then clamped onto the spacers, and the excess vaseline removed. The separating gel was then poured into the gap between the plates and immediately overlaid with water (approx 0.5cm) and allowed to set for 45 minutes. Once polymerisation had occurred, the water was drained off.

The stacking gel solution was then made up by dissolving 10g of acrylamide, 0.27g of bisacrylamide, 0.6ml of TEMED, 3.0g of TRIS and 4ml of glycerol in 200ml of distilled water adjusted to pH 6.8 with HCl.

10 μ l of 10% w/v ammonium persulphate was added to 4ml of the stacking gel, the comb was then slipped into the plate and the stacking gel was pipetted around it, the top of the stacking gel was then overlaid with water and left to polymerise. Once polymerisation of the stacking gel had occurred, the comb, clamps and bottom spacer were removed and the plate was clamped in the gel tank. The running buffer was then made up by dissolving 114g glycine and 30g of TRIS in 1 litre of distilled water, this was then diluted (ten fold) and poured into the upper and lower reservoirs. A syringe with a bent needle was used to wash out the air bubbles trapped between the plates and a pasteur pipette was used to wash out the sample wells.

The protein samples applied were adjusted to concentrations of 125 μ g/ml or 187.5 μ g/ml for each sample. These were made up in sample buffer, which consisted of 10ml glycerol, 5mM mercaptoethanol and 0.76g TRIS dissolved

in 100ml of distilled water adjusted to pH 6.8 with HCl containing 6ml of 2% bromophenol blue. The samples were then trickled into the wells and the apparatus run at 40 volts for two hours and then 25 volts overnight. (The methods used above were obtained from the LKB laboratory manual 2001-000-1ME).

2.6 Inhibitor Studies on *Penicillium cyclopium* using Clofibrate

Forty flasks of *P.cyclopium* were grown as described in 2.2b. 24 hours after the primary inoculum had been homogenised and used as a subculture for the 40 flasks, 2-(p-chlorophenoxy)-2 methylpropionic acid (Clofibrate) was added to 32 of the flasks. Clofibrate was made up at 100mg/ml in saturated sodium bicarbonate (0.46M Clofibrate). The 32 flasks that had had clofibrate added were treated as follows, 8 flasks at 46mM per flask, 8 flasks at 4.6mM per flask, 8 flasks at 0.46mM per flask and 8 flasks at 0.046mM per flask, the remaining flasks were used as blanks. 24 hours after the Clofibrate was added (day 2 of growth) 2 of the flasks from each set were removed as samples and Clofibrate was added to the remaining flasks at its respective concentration. Similarly on day 3 and 4 samples were taken and more Clofibrate added at its respective concentration to the remaining flasks. On day 5 the last samples were taken.

The metabolites were extracted (section 2.3b), partially purified on an XAD-2 column (section 2.3b.2.) and separated by t.l.c. on silica gel plates (section 2.3b.1.) for assay.

2.7 Protein Determination

Protein was routinely determined using u.v. absorbance, where the protein concentration is found by the equation:

Method 1

$$\text{Concentration(mg/ml)} = 1.55A_{280} - 0.76A_{260}$$

The above equation was suggested by Lowry and published by Kalckar (1947), and is derived from the data of Warburg and Christian (1941). A second method was used to check these results (Groves, Davis and Sells 1967), in this method the absorbance of the sample was found at 224nm and 233nm. The concentration was then found from:

Method 2

$$A_{224} - A_{233}$$

The resulting absorbance was then related to the calibration curve:

Change in Absorbance	0	0.25	0.5	0.75	1.0
$A_{224} - A_{233}$					
Concentration ($\mu\text{g/ml}$)	0	50	102.5	155	207.5

The methods of protein determination were chosen for the following reasons; they allow subsequent use of the protein sample and using the calibration curve in the case of method 1, interference due to nucleic acids can be eliminated (only up to 20% nucleic acid), method 2 is designed to account for the absorbance due to nucleic acids, both of these methods can be used down to the microgram range.

The draw backs are:

The absorbance value is effected at pH's other than 7, nucleic acid concentrations over 20% will affect the readings (nucleic acid was found from the 260 / 280 readings and was rarely above 4%). With method two, compounds containing two conjugated double bonds would interfere with the assay, however these compounds would be unlikely to appear in these experiments in large quantities.

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The following popular methods of protein determination were not used for the reasons given:

1. The Folin - Lowry method (Lowry et al 1951) is sensitive in the microgram range and is relatively unaffected by nucleic acid but is inaccurate in the presence of $(\text{NH}_4)_2\text{SO}_4$, guanine, glycine, α - and β -CA and some other biological materials (Peterson 1979).
2. The Biuret method is sensitive only in the milligram range and is inaccurate in the presence of $(\text{NH}_4)_2\text{SO}_4$ and Mg^{2+} ions (Layne 1957).
3. The Micro Biuret method (Zamenhof 1957) is sensitive in the microgram range, but any chelators will affect the method, and α - and β -CA are good chelators.
4. The Kjeldahl method, is not only time consuming but ammonium ions and nucleic acids and in fact any nitrogen source interferes with the protein determination.
5. The turbidity determinations, any precipitable non - protein material will give erroneous results.

CHAPTER 3

RESULTS AND DISCUSSION

THE ENZYMES

3.1 Extraction and Purification of Enzymes from *Penicillium cyclopium* and Pig Liver

Pig liver and *P. cyclopium* were extracted as previously shown (2.5.1) and the enzymes assayed (2.4). The enzyme activity is shown in Table 1. The isomerase of pig liver was isolated first, following the procedure of Holoway and Popjak (1968) as the isomerase was needed to test for the transferase.

The initial protein concentration of the supernatant after centrifugation at 48000g was always measured, as the concentration of ammonium sulphate required to precipitate the protein is dependant on the protein concentration, as well as the temperature. As these fractionations were carried out at 5°C the conversion nomograph of di Jesso was used. The activities from the ammonium sulphate fractionations of pig liver are shown in Table 2.

The ammonium sulphate fractions were dialysed against extraction medium to remove the ammonium sulphate, and then loaded on to an Ultrogel Aca 34 column (See Figures 6 and 7). The protein sample was loaded onto the column at 3-6% of the column volume (6% is 20mg of protein). It is known (Holoway & Popjak 1967 & 1968) that for pig liver fractions 50-70% contain mainly isomerase and fractions 40-60% contain mainly transferase. They showed that when eluted from a Sephadex G200 column the isomerase was eluted after the haemoglobin and the transferase before the haemoglobin. My results confirm this. Using the Ultrogel Aca 34 column total separation of the isomerase from the transferase was possible for both pig liver and *P. cyclopium*. Figures 6 to 9 show the separation achieved on an Ultrogel Aca 34 column using both ammonium sulphate fractionated, and non fractionated samples. It can be seen from Figures 6 and 9 that both the isomerase and the primary transferase are separated extremely well by size, and very little overlap of the enzymes occurs. It was therefore felt

TABLE 1 Assay of primary transferase activity of crude and centrifuged extracts of pig liver.

steps	dpm	nM IPP utilised /min	Specific activity
crude extract	29294	6.38	0.0019
centrifuged 3 hours at 48000g	27189	5.93	0.002

TABLE 2 (A) Assays of ammonium sulphate fractions of the isomerase from pig liver.

% ammonium Sulphate	dpm	n mole IPP utilised / min	enzyme units	protein conc in mg/ml	Specific activity
0-30	3336	0.595	0.0006	66	0.0009
30-40	6565	1.1724	0.001172	44.5	0.00261
40-50	26802	4.788	0.004788	43.5	0.011
50-60	35969	6.42	0.00642	49.5	0.0129
60-70	7382	1.32	0.00132	41.5	0.00318

(B) Assay of ammonium sulphate fractions of the primary transferase from pig liver. (corrected for isomerase blank)

% ammonium Sulphate	dpm	n mole IPP utilised / min	enzyme units	protein conc in mg/ml	Specific activity
0-30	2774	0.496	0.000496	66	0.000752
30-40	21156	3.78	0.00378	44.5	0.00849
40-50	8153	1.452	0.00145	43.5	0.00334
50-60	1615	0.29	0.00029	49.5	0.000586
60-70	1186	0.216	0.000216	41.5	0.00052

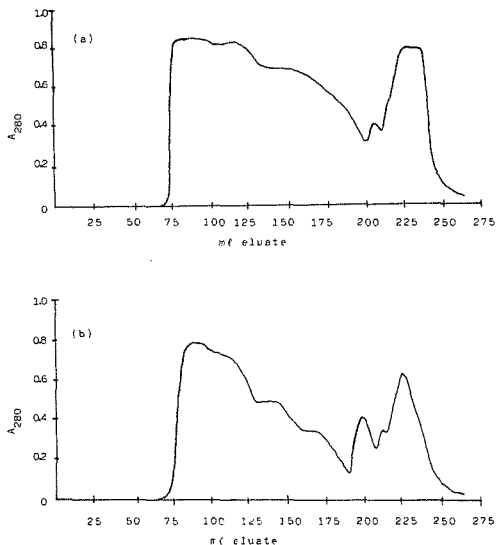


Figure 6. Elution profile of the 30 - 40% and 50 - 60% ammonium sulphate fraction from pig liver. : The 30 - 40 (a) & 50 - 60% (b) ammonium sulphate fraction from pig liver eluted from an Ultrogel AcA 34 packed column 100 x 2.5cm. The peak seen at 140 - 175ml is transferrase, the peak at 190 - 215ml is haemoglobin and the peak at 225 - 260ml is isomerase. Absorbance was measured by flow through meter at 280nm.

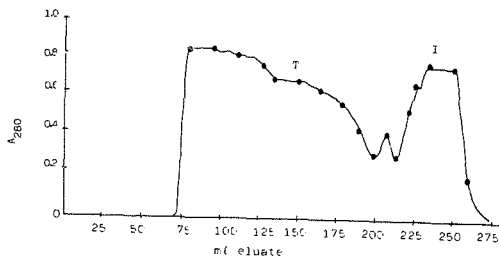


Figure 7. Elution profile of the centrifuged unfractionated pig liver sample: The protein sample, centrifuged but not fractionated with ammonium sulphate was eluted from an Aca 34 Ultrogel packed column 100 x 2.5cm. The peak seen at 160ml is transferrase, the peak at 200ml is haemoglobin and the peak at 225ml is transferrase. Absorbance was measured by flow through cell at 280nm.

in further separations that ammonium sulphate fractionation was not necessary as careful collection of the fractions gave isomerase and primary transferase fractions not contaminated by each other.

Holloway and Popjak also showed that the isomerase has a distinct pH optimum at 6.0 and is activated by Mn^{2+} in preference to Mg^{2+} . This property is only seen after dialysis of the enzyme against EDTA and repeated dialysis against a medium free of EDTA, suggesting that the Mn^{2+} is firmly bound to the native enzyme. Shah (1965) working with pig liver obtained a crude extract in which the isomerase showed a preference for Mg^{2+} , however it is thought that this was probably due to the presence of a phosphatase in the extract. My work, unreported here, showed that the isomerase isolated from pig liver according to the method of Holloway and Popjak (1967 & 1968) showed a preference for Mn^{2+} and that this was also the case for the isomerase isolated from *P.cyclopium*.

Holloway and Popjak (1968) also showed that the equilibrium of the reaction favoured the production of dimethylallylpyrophosphate, in the ratio of 3:1, and that the reaction is reversible. Taking this as a fact (it is also supported by Lehninger 1975, and Conn and Stumpf 1976), the isomerase from pig liver was used in the enzyme assays to make [1- ^{14}C] DMAPP and was used in both the pig liver and *P.cyclopium* assays.

With the dimethylallylpyrophosphate transferase, they found the enzyme was equally active with either DMAPP + IPP or geranyl pyrophosphate + IPP as substrates, and whichever was used the product was always farnesyl pyrophosphate. The pH optimum of the enzyme was found to be 7.9 and requires Mg^{2+} ions as activators in preference to Mn^{2+} . The preference for Mg^{2+} over Mn^{2+} for activation was also seen in this study for *P.cyclopium*.

Cell Disruption

The three methods of cell disruption which were investigated in the extraction of protein from the "mets" of *P.cyclopium* were the Waring blender (2 min) the Potter-Elvehjem homogeniser (4 min) and the acetone/dry powder method. These results are shown in Table 3 and the best method of disruption was acetone/dry powder. It was found to be the best method not only for the ease of isolation of the enzymes, and the increased amount of enzyme liberated (seen by the increase in activity Table 3), but also because the use of acetone removed many of the fats released during the crude isolation, and the powder was easy to store and showed high activity when reconstituted (results not shown). Approximately 480gm of wet *P.cyclopium* from all 24 flasks yielded approx 30-35gm of powdered *P.cyclopium*.

In each of the experiments 5gm of acetone/dry powder was used, the powder was suspended in extraction medium and then centrifuged at 48000g for 3 hours. In some experiments the supernatant was fractionated with ammonium sulphate, in others it was applied to the Aca 34 column without fractionation. In the case of the fractionated samples the pellet (after centrifugation) was suspended in extraction medium and dialysed for 4-5 hours.

The activity shown in the initial extracts, before fractionation, are shown in Table 4, and the activity shown after ammonium sulphate fractionation is shown in Table 5. Comparing Tables 2 and 5 it can be seen that the isomerase and transferases from *P.cyclopium* follow the same distribution as those from pig liver, and show the same molecular weights when run on an Aca 34 column (the elution times and volumes being the same) and when kept under the same storage regimes, keep very well, at least 18 months at -70°C and 6 months at -20°C. It was also found in this study that the optimum transferase activity from both the pig liver and *P.cyclopium* was found in the 30 - 40% ammonium sulphate fractionation range, and the isomerase in the 50 - 60% ammonium sulphate fraction.

TABLE 3 Assay of the primary transferase for the three methods of cell disruption used with *P. cyclopius*

Method	dpm	Specific activity
Waring blender 2 minutes	591	0.00006
Potter-Elvehjem homogeniser 4min	1282	0.0001
Acetone/ dry powder	2775	0.00013

TABLE 4 Assay of the primary transferase from *P. cyclopius* from a crude and centriuged extract.

Steps	dpm	nM IPP utilised /min	Specific activity
crude extract	23425	5.11	0.0013
centrifuged 3 hours at 48000g	21800	4.757	0.0016

TABLE 5 (A) Assays of the ammonium sulphate fractions of the isomerase from *P. cyclopius*

% ammonium sulphate	dpm	n mole IPP utilised / min	enzyme units	protein conc in mg/ml	Specific activity
0-30	1927	0.48	0.00048	18.4	0.00261
30-40	2320	0.588	0.000588	9.8	0.006
40-50	8214	2.112	0.002112	10.4	0.0204
50-60	13904	3.564	0.00356	11.3	0.03154
60-70	3718	0.984	0.000948	9.3	0.0102

(A) Assay of the ammonium sulphate fractions of the primary transferase from *P. cyclopius*. (corrected for isomerase blank)

% ammonium sulphate	dpm	n mole IPP utilised / min	enzyme units	protein conc in mg/ml	Specific activity
0-30	4102	0.732	0.000732	18.4	0.003978
30-40	8558	1.528	0.001528	9.8	0.01559
40-50	5917	1.058	0.001058	10.4	0.01022
50-60	2085	0.372	0.000372	11.3	0.00329
60-70	832	0.148	0.000148	9.3	0.001591

Holway and Popjek found the transferase in the 3S - 45% fraction and the isomerase in the 60 - 70% fraction, the discrepancy though is probably due to the initial protein concentration, as the percentage ammonium sulphate used to precipitate a certain protein is proportional to the protein concentration.

In experiments with both pig liver and *P.cyclopium* enzymes the isomerase was isolated first as has been mentioned. In the cases where the unfractionated sample was added to the ACA34 column, isomerase previously isolated from pigs liver was used in assays for both the pig liver and *P.cyclopium* transferases (See Figures 8 and 9). This was done as only [$1\text{-}^{14}\text{C}$] IPP was available, and the IPP had to be converted to DHAPP using the isomerase before it could be used as a substrate for the transferase.

Mc Grath (1977) used an ice shear to disrupt the cells in his work. However he found that when run on a column to check the molecular weights, the molecular weights varied from experiment to experiment. He also found that when he attempted to separate the enzymes using an ion exchange column that the enzymes were highly unstable and did not remain active, the same was found with isoelectric focusing. It was therefore decided in these experiments not to use the ice shear as it was thought that there may be some pressure effect causing dissociation of the enzyme subunits (J.Davis personal communication), or possibly dissociation followed by faulty re-association as Mc Grath noted that some activity remained for a short time after the end of his purification experiments. It should be noted that powder extracts of *P.cyclopium* when stored at -20°C retained their activity for at least a year, and after DEAE chromatography, gel electrophoresis and isoelectric focusing retained their activity for at least 24 hours. (Samples kept from DEAE were active for many weeks if stored at -20°C).

Powdered *P.cyclopium* was prepared from *P.cyclopium* grown on NaNO_3 and corn steep liquor (CSL) as nitrogen source. The enzymes of secondary metabolism

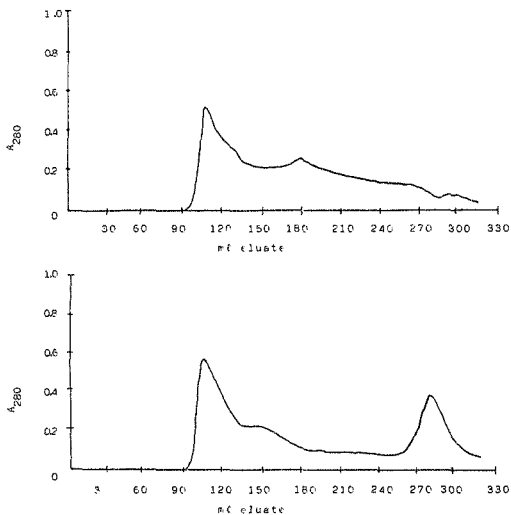


Figure 8. Elution profile of the 30 - 40 and 50 - 60% ammonium sulphate fractions from *P. cyclopius*: The 30 - 40% (a) and 50 - 60% (b) ammonium sulphate fractions from *P. cyclopius* were eluted from an Ultrogel ACA 34 packed column, 100 x 2.5cm. The peak at 180ml showed transferase activity and the peak at 290ml showed isomerase activity. Absorbance was measured by flow through cell at 280nm.

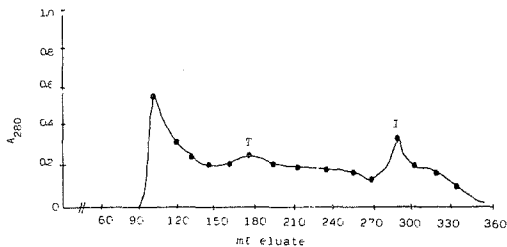


Figure 9. Elution profile of the centrifuged unfractionated protein sample from *P. cyclopius* : The protein sample was the centrifuged unfractionated sample from *P. cyclopius* and was eluted from an Ultrogel ACA 34 packed column 100 x 2.5cm. The peak at 180ml shows transferase activity and the peak at 295ml showed isomerase activity. Absorbance was measured by flow through cell at 280nm.

from *P.cyclopium* grown on both CSL and NaNO_3 (see section 3.5 pg.48) were isolated in the same way as each other, and showed no differing characteristics. Therefore if enzymes are needed it is preferable to grow the organism on CSL as the yield of mycelia is so much higher, almost 2gm/100ml of culture medium as against 0.29gm/100ml when grown on NaNO_3 and thus the yield of enzymes is higher. However the yield of secondary metabolites is lower, and so to obtain secondary metabolites the organism must be grown on NaNO_3 as nitrogen source.

It was found to be more expedient in the case of both *P.cyclopium* and pig liver extracts, not to fractionate with ammonium sulphate, but to add aliquots of the centrifuged extract to the column. In this way a large stock of semi-purified enzyme was built up either for further purification or for use in assays. The primary transferase and isomerase from both *P.cyclopium* and pig liver eluted from the Ultrogel column at approximately the same volumes, when the unfractionated centrifuged sample was added to the column. When the isomerase and primary transferase from both sources were fractionated they each precipitated out at the same respective ammonium sulphate concentrations.

3.2 DEAE Cellulose Fractionation of *Penicillium cyclopium* and Pig Liver Enzymes

This was carried out for both *P.cyclopium* and pig liver enzymes as described in 2.5.6. The centrifuged cell free extract samples from both were added separately to the column. The partially-purified pig liver isomerase from the Aca 34 column was added in an attempt to purify it further. The partially purified *P.cyclopium* sample was of isomerase. The 100 & 150 mM NaCl fractions derived from the ion exchange chromatography of the *P.cyclopium* extract, were found to contain isomerase. These were dialysed against extraction medium for 24 hours followed by dialysis against solid sucrose to concentrate them, and then stored in 1ml aliquots at -70°C . The partially-purified pig liver isomerase was used in experimental assays to test for the transferase from *P.cyclopium* and pig

TABLE 6 DEAE fractionation of the Isomerase and Transferase of *P. cyclopius* and Pig Liver separated on a DE 11 column, elution being effected with NaCl.

(A) 250mg crude non ammonium sulphate fractionated sample from pig liver, assay for the transferase

Concentration in mM NaCl	dpm	Specific activity
0	20	0.0000
50	1024	0.0069
100	2468	0.265
150	1528	0.096

(B) 75mg partially purified (from the Ultrogel AcA34 Column) isomerase sample from pig liver

Concentration in mM NaCl	dpm	Specific activity
0	6	0.0000
50	1310	1.12
100	1710	2.83
150	2014	1.63

(C) 200mg crude non ammonium sulphate fractionated sample from *P. cyclopius*, assay for the transferase

Concentration in mM NaCl	dpm	Specific activity
0	0(-24)	0.0000
50	1064	0.05
100	1752	0.253
150	1328	0.176

(D) 75 mg partially purified (from the Ultrogel AcA34 column) isomerase sample from *P. cyclopius*

Concentration in mM NaCl	dpm	Specific activity
0	12	0.0000
50	1121	0.547
100	2156	2.43
150	2213	1.09

liver. Both the *P.cyclopium* and pig liver isomerases and transferases eluted from the column at the same NaCl concentrations, and kept for at least 3 months at -20°C . (See Table 6).

3.3 Polyacrylamide Slab Gel Electrophoresis of the Isomerase of *Penicillium cyclopium* and Pig Liver

Slab gel electrophoresis was carried out as described in 2.5.8. The results are shown in Figure 10. The sieving effect of the gels is a critical factor in separation. The density of the gel network (pore size) is varied according to the size of the protein sub-units being separated (Maurer, M.R., 1971; Work, T.S. and Work, E., 1969). In this case, the gel used was 10% (T) giving a molecular weight fractionation range of 10 000 - 80 000 daltons. It should also be remembered that the pH of the system must be chosen with care as the pH of the buffer greatly affects protein mobility (i.e. the charge the protein is carrying).

The isomerases from both *P.cyclopium* and pig liver were used in this experiment and partial purification of the enzymes was obtained for the unfractionated crude extracts. The partially purified pig liver isomerase from the DEAE cellulose fractionation (150 mM NaCl) showed three bands. The main band being the isomerase, the others showed no activity in the assays. However using any of the mentioned techniques it was impossible to remove the contaminating bands from the isomerase. Figure 10 a and c show the attempted separation of the crude centrifuged extract of *P.cyclopium* and pig liver. It can be seen that partial purification occurred. However the results obtained did not warrant the use of this method as a purification step as it is long and can only deal with small amounts of material. The enzymes retained their stability through this procedure, as when eluted from the PAGE slabs they were found to remain active for up to 3 months if stored at -20°C (unpublished results).

crude	0	50	100	150
1	1	1	1	1
2	1	1	1	1
3	1	1	1	1
4	1	1	1	1
5	1	1	1	1
6	1	1	1	1
7	1	1	1	1
8	1	1	1	1
9	1	1	1	1
10	1	1	1	1
11	1	1	1	1
12	1	1	1	1
13	1	1	1	1
14	1	1	1	1
15	1	1	1	1
16	1	1	1	1
17	1	1	1	1
18	1	1	1	1
19	1	1	1	1
20	1	1	1	1
21	1	1	1	1
22	1	1	1	1
23	1	1	1	1
24	1	1	1	1
25	1	1	1	1
26	1	1	1	1
27	1	1	1	1
28	1	1	1	1
29	1	1	1	1
30	1	1	1	1
31	1	1	1	1
32	1	1	1	1
33	1	1	1	1
34	1	1	1	1
35	1	1	1	1
36	1	1	1	1
37	1	1	1	1
38	1	1	1	1
39	1	1	1	1
40	1	1	1	1
41	1	1	1	1
42	1	1	1	1
43	1	1	1	1
44	1	1	1	1
45	1	1	1	1
46	1	1	1	1
47	1	1	1	1
48	1	1	1	1
49	1	1	1	1
50	1	1	1	1

(c)

Figure 10. Polyacrylamide gel electrophoresis of pig liver and *Penicillium cyclopium* samples after DEAE fractionation. : Line diagrams of polyacrylamide gel electrophoresis of (a) pig liver isomerase, unfractionated, from DEAE cellulose, (b) pig liver isomerase from a gel filtration partial purification, and (c) *P. cyclopium* isomerase, unfractionated, from DEAE cellulose. The numbers at the head of each gel indicate mM concentrations of NaCl. See Table 6 for the enzyme activities at the various salt concentrations.

3.4 Isoelectric Focusing of the Enzymes of *Penicillium cyclopium*

Isoelectric focusing was carried out (as in 2.5.7) using the protein from reconstituted acetone/dry powder of *P. cyclopium*. Figure 11 shows the uniformity of the pH gradient, the correlation coefficient was $r = 0.98103$ (26 readings).

All the segments of gel were tested for activity using the enzyme assays, thereafter only segments showing bands were tested for activity. The isoelectric points for the transferase and isomerase were found to be 5.2 and 4.9 respectively. The p.I.'s were checked over 3 successive runs and were not found to differ. ^{M^C} Grath *et al* (1977) found that when they attempted isoelectric focusing, using an upright LKB column, the enzymes did not survive for more than 1 hour after the run. This was possibly due to damage caused by pressure in the ice shear method of cell disruption. cAATrp was needed to assay for the secondary transferase, and problems were being encountered in the isolation and purification of this substrate. A small amount of cAATrp had been synthesised (P. Steyn CSIR) and although old and slightly impure (showed 2 or possibly 3 bands when checked by t.l.c.) was used to assay for the secondary transferase after its isolation using isoelectric focusing and gave a pI of 5.6. ^{M^C} Grath *et al* (1977) found pI's slightly different (I = 4.5; T = 5.1; S = 5.3) to those reported here, possibly due to his use of an upright column, causing some movement of the protein bands at the end of the run. They also used an ice shear technique to obtain their cell free extract, and it is possible that this caused a pressure jump effect (Davis ; ^{M^C} Grath, personal communication) leading to the dissociation of the sub-units of the enzyme, thus causing instability of the protein complex.

Isoelectric focusing was carried out on the centrifuged extract of *P. cyclopium*. An apparatus was fabricated to utilise a Bio-Rad electrofocusing plate. 10 large bands were found on the gel using fluorescent silica gel strips, of these 7 were visible to the naked eye. There were 6 main bands and 4 subsidiary bands, one of the visible main

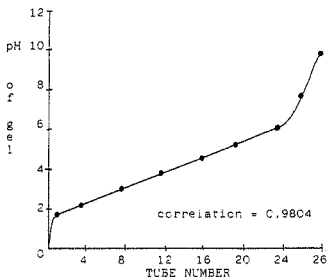


Figure 11. pH Gradient of the Isoelectric Focusing Gel: The isoelectric focusing gel was divided into 26 equal segments (1.15cm). The segments were suspended in distilled water and the pH read from tubes 2 - 24 and the gradient was seen to be unit/cm, showing a correlation coefficient of 0.9804. Enzyme activity was seen at pH 4.9 for the isomerase and pH 5.2 for the primary transferase. Secondary transferase activity was seen at pH 5.6

bands was the transferase and one of the larger subsidiary bands, not visible to the naked eye, was the isomerase.

The visible bands could be used to check the uniformity of the run by their straightness. It also facilitated the removal of the bands for assaying as the bands not visible to the naked eye followed the contours of the visible bands. The bands were also useful in determining the accuracy of the run, for if the electrodes had been incorrectly fitted then the bands became distorted over the length of the gel bed.

3.5 Production of the Secondary Transferase by *Penicillium cyclopium* in Minimal and Rich Media

During the phase of rapid growth of *P. cyclopium* small amounts of CA are produced, however a sharp increase in the rate of CA production is seen following the decline in the activity of the primary metabolic process. The decline in primary metabolism and the attendant acceleration in CA production are related to the depletion of nutrients (Neethling 1972). In these experiments the nitrogen source in the medium was the limiting factor.

Secondary metabolism and active growth are generally considered to be competitive and mutually exclusive processes. Bu'Lock (1965) has suggested that the concentration of various intermediates increases rapidly when active growth is terminated and that secondary metabolic pathways are "switched on" fully, to alleviate this build up, meaning that once the substrates are available, the enzymes involved are synthesised. Thus the increase in the rate of CA production when primary metabolism is declining may be due to the increased availability of tryptophan or dimethylallylpyrophosphate. This could lead to accelerated CA production (Neethling 1972). Luckner (1975) has suggested that the enzymes are produced before they are needed, or at least before the substrates become available to these enzymes. It is known with *P. cyclopium* that the secondary transferase is produced early (day 2-3) and is in fact depleting

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when maximal CA production occurs. The secondary transferase appears at the same time as the primary transferase in *P.cyclopium* and may therefore be on the same operon. If this were so then the secondary transferase may well be produced independently of the type of medium (either well-defined or rich), in other words the secondary transferase may be produced independently of CA production

In order to investigate this *P.cyclopium* was grown on two nutrient sources where it is known (Neethling 1972) that *P.cyclopium* grown on NaNO_3 as nitrogen source produces large amounts of CA (99.08mg/gm mycelium) but the least mycelium (0.29gm/100ml culture medium). Whereas if *P.cyclopium* is grown on CSL the yield of CA is low (8.85mg/gm mycelium) but mycelial production is high (2gm/100ml culture medium).

A solution of cAATrp was prepared containing 75.1ug/ml cAATrp (10.8mt) which when run on a t.l.c. plate displayed one major band with one minor band immediately below it. This cAATrp was then used in an assay using enzyme extracts from *P.cyclopium* (ie the dried powder resuspended in extraction buffer and centrifuged at 48000g for three hours, to provide a clear supernatant solution containing the primary and secondary transferase's and the isomerase). The use of the purified cAATrp in the secondary transferase assay confirms its standing as a substrate of the secondary transferase as in use with $[1-^{14}\text{C}]$ IPP only B-CA was labelled. The results of the enzyme assay using cAATrp are shown in Table 7, where identical procedures were followed for the treatments to the secondary transferases grown on both NaNO_3 and CSL.

In tube 1 and 2 after incubation for 5 min, the reaction was stopped with HCl and each tube extracted with 1ml of chloroform containing 1% α - and B-CA and cAATrp; 500 μ l of the chloroform from each tube was then layered onto t.l.c. plates and developed. The silica gel bands containing α - and B-CA were scraped off and tested for radio-activity. Tubes 3 and 4 were treated in the same way except the intermediates were extracted from the tubes using 1% of each of farnesol; nerolidol and geraniol in chloroform

TABLE 7 Enzyme assay of the secondary transferase with cAAtrp.

The assay was carried out on enzyme extracts grown on two media:

1: Medium with NaNO_3 as nitrogen source

2: Medium with CSL as nitrogen source

The amounts are shown below;

Tube	Incubation medium(ml)	Purified cAAtrp(ml) (75.1 μ g/ml)	Crude enzyme	dpm	
				NO_3	CSL
1	0.8	0.1	0.0	270	191
2	0.8	0.1	0.1	2469	2482
3	0.8	0.1	0.0	219	152
4	0.8	0.1	0.1	445	254

Tube 1 & 2 : Incubation medium : pH 6.5 (10mM) TRIS HCl; 50 μ mole $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$; DTE 1mM; 59.43 n mole $[1-^{14}\text{C}]$

IPP

Tube 3 & 4 : Incubation medium : pH 7.6 (10mM) TRIS HCl; 50 μ mole $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; DTE 1mM; 59.43 n mole $[1-^{14}\text{C}]$

IPP

Tube 1 is the blank for tube 2, which shows the secondary transferase activity at pH 6.5, where the isomerase is most active. Tube 3 is the blank for tube 4, which shows the activity of the secondary transferase at pH 7.6, where the isomerase is almost inactive. The isomerase is used to produce DMAPP, one of the secondary transferases substrates.

; for tube 3 the 500 μ l was added straight to scintillation fluid; for tube 4 the 500 μ l was plated out and run on t.l.c. plates. The α - and β -CA was then scraped off and tested for radio-activity.

These results show that the secondary transferase from *P.cyclopium* grown on CSL is present and is as active as the secondary transferase from *P.cyclopium* grown on NaNO_3 . The concentration of the enzyme is low due to the amount of dilution involved in the assay. As it has been previously found (Neethling 1972) that *P.cyclopium* grown on CSL causes the greatest mycelial growth (2gm/100ml) but the least CA yield (8.85mg/gm mycelium) whereas *P.cyclopium* grown on NaNO_3 causes the least mycelial growth (0.29gm/100ml) but the greatest CA production (99.08mg/gm mycelium).

It is therefore important to note that as larger masses of *P.cyclopium* can be grown up on CSL and that the same amount of secondary transferase activity is found in the same mass of dried mycelia that this method of growth allows for the extraction of large amounts of secondary transferase from *P.cyclopium*. From Table 7 it can be seen that if a correction is made for the blank the activity of CSL extracted enzymes is the same as that of the enzymes extracted from NaNO_3 as nitrogen source. As a 77 fold decrease in CA production is known (Neethling 1972) to occur in *P.cyclopium* grown on CSL as opposed to NaNO_3 and yet the levels of the secondary transferase, which is responsible for CA production, are the same. This is suggestive that some factor other than the amount of enzyme is responsible for the decreased CA production found in *P.cyclopium* grown on CSL.

The control of secondary metabolism has recieved some study, but very little is still known of the control points. Bu'Lock (1980) made a general observation that in many processes of primary metabolism the overall kinetics are normally governed by substrate level, because the enzymes are usually present in excess. Secondary metabolism is usually however enzyme limited, possibly because of the position of secondary metabolism

in the general economy of the cell, or because of the manner in which secondary metabolic pathways branch off from the primary network.

Luckner *et al* (1977) demonstrated that in microorganisms and higher plants the activities of enzymes involved in secondary metabolism appear before or during the period of secondary product biosynthesis, and that expression depends on the *de novo* synthesis of RNA and proteins. Luckner goes on to say that although regulation of the amount of enzyme plays a part in the regulation of secondary metabolism, regulation of the enzyme activity may be as important. Luckner *et al* (1977) suggests compartmentation of the enzymes or the production of enzyme which is immediately rendered inactive either by the binding of co-factors, enzyme-enzyme binding or the binding of enzyme to organelle membranes as the primary control of secondary metabolism.

Bu'Lock (1980) on the other hand agrees only with Luckner in that the control of enzyme synthesis is important. Bu'Lock states that controlling mechanisms which act at the level of gene expression, and govern *de novo* enzyme synthesis are probably the most important control points in secondary metabolism. In effect, during secondary metabolism, under certain nutrient shortages, the microorganism expresses a series of genes which are silenced under optimum growth conditions.

Such a control mechanism can be found in bacteria and is known as "catabolite repression". In bacteria this controls the initiation of gene transcription by providing common control for a large number of unlinked gene sites. In this system each individual site can still be subject to more specific controls such as substrate or product linked derepression or induction.

The production of enzymes leading to the formation of α - and β -GA in *P.cyclopium* grown on nitrogen limited medium (NaNO_3 minimal medium, see 2.1(A)) is well known. The production of an active secondary transferase from *P.cyclopium* grown on CSL (which is not nitrogen limiting) has now

however been shown. Therefore Bu'Lock's hypothesis that the production of the active enzyme is dependant on nutrient shortage causing the gene for the secondary transferase to be derepressed obviously does not occur in *P.cyclopius* for the production of α - and β -CA. Similarly the enzymes used in the above experiment were from a centrifuged cell free extract. As such it would be expected that any co-factors attached to the enzymes, or covalent or hydrogen bonds formed between enzymes would remain intact, and any enzyme bonded to organelle membranes would be centrifuged out. Therefore according to Luckner most of the secondary transferase found in the *P.cyclopius* grown on CSL would be in the inactive form (or centrifuged down and discarded attached to organelle membranes). However when the above experiment was carried out, using crude secondary transferase from *P.cyclopius* grown on both CSL and NaNO_3 , it was found that the enzymes had the same activities under identical conditions. This suggests that some factor other than compartmentation and inactivation or repression of the genes concerned is responsible for the inhibition of the secondary transferase in *P.cyclopius*.

It can now be seen that the secondary transferase is produced in an active state under conditions of no nutrient stress in *P.cyclopius*. Perhaps an alternative hypothesis to those of Bu'Lock and Luckner would be that, although the secondary transferase is produced in an active form, when primary metabolism is under no stress, competition by secondary metabolism for substrates from primary metabolism is low. Yet when primary metabolism is under nutrient stress it is easier for the enzymes of secondary metabolism to obtain their substrates from primary metabolism and secondary products are formed.

THE METABOLITES

Cyclopiazonic acid has been previously isolated by Holzapfel (1968), using a cellulose packing and eluting with a gradient using 3.5M formic acid in 1:1 aqueous methanol, as can be seen a very high ionic strength solution was needed to elute the CA. Similarly in the following experiments using XAD-2, silica gel and Sephadex G10 it was found to be extremely difficult to remove the CA from the packing ie. 100% methanol, 100% propionic acid and 2.5M ammonium formate had no effect on the elution of CA, and eventually it was found to be necessary to use 50% acetone at pH 2 to break the bonds holding the CA to the column packing. Hydrogen bonding is therefore probably responsible for this binding, because 50% acetone at pH 2 is a well known H-bond disruptor. Although for the separation of CA, cAAtrp, trp-y and trp-z the strong binding is a problem, it is interesting to speculate as to whether the strong binding of CA to the carbohydrate in the column packings is related to the way in which CA brings about death in animals ie inhibiting carbohydrate movement or by chelating Mg^{2+} or Mn^{2+} .

It was found that if samples of the fractions containing CA, cAAtrp, trp-y and trp-z were run on t.l.c. plates after separation, that various bands appeared, instead of a single band for each compound. The groups of bands each stained the correct colour with Ehrlich reagent, and had R.F. values in close proximity to each other. It is known that CA has four possible stereoisomers, and in all the run plates CA appeared with two bands at each of the a- and B- positions and in most cases appeared to show three or four bands for each type (see Figure 12).

Throughout cAAtrp has shown two distinct bands, as have trp-y and trp-z. It was also noticed that many (between 5 and 13) bands were seen between a-CA and tryptophan at the base line, suggesting many more unknown intermediates or isomers of intermediates. Time permitting it would have been interesting to isolate these intermediates in quantities large enough to allow their structures to be elucidated and to attempt

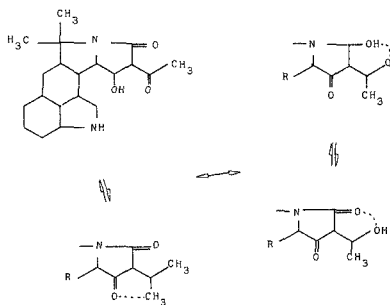


Figure 12. Tautomers of Cyclopiazonic acid

re-feeding experiments in both growing cultures and with isolated enzymes.

3.6 Chromatography of the Metabolites of *Penicillium cyclopium* in CA Production

Cultures were grown on sodium nitrate medium for five days, both the aqueous and organic layers were taken and the metabolites extracted (2.3a). These metabolites (now in one ml aliquots) were used on the columns, a small portion of the samples being run on a t.l.c. plate to check the presence of all metabolites. Three column packings were examined, (i) XAD-2, (ii) Silica gel and (iii) Sephadex G10.

Initially an XAD-2 packing was used, XAD-2 is a non-polar adsorbant prepared by copolymerisation of styrene and divinylbenzene, it forms very porous microspheres, but penetration is low and most of the adsorption phenomena take place on the surface. The adsorption effects are based on van der Waals interactions between the hydrophobic part of the molecule and the non-polar matrix. Adsorption is stronger the more polar the adsorbant and hence substances are very strongly adsorbed from water (anonymous 1971). It has been used for example in the separation of aqueous solutions of meat flavour precursors (Zaika *et al* 1968), for the elimination of excess picric acid used in the deproteinization of cell extracts (Zaika 1969) and the separation of nitro- and chlorophenols (Grieser 1973), and in the partial purification of transferase from pig liver (Holoway & Popjak 1967). In this work methanol was used to wash metabolites off the column, however this technique proved unsatisfactory as the CA washed off the column indiscriminately and thus contaminated all the fractions even though separation appeared to occur (see Figs 13, 14 & 15). It was then decided to use a Silica gel column as previous work with t.l.c. plates had shown good separation. However it became obvious that the methods of separation in the column and on the plate differed as no separation was possible on the silica gel column. It is possible that this lack of success using the silica gel column is due to the fact

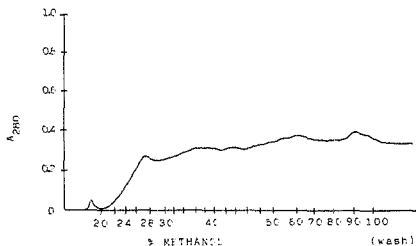


Figure 13. Elution profile of a shallow gradient from an XAD-2 column: In an attempt to separate cAatrp from the other secondary metabolites of *P. cyclopium* Amberlite XAD-2 was used as the packing. The sample was added to the column (30 x 15cm) via an LKB vario-perpex pump. The above column was eluted with 20ml aliquots from 20 - 50% methanol in stepwise increases of 2%. α - and β -CA were found in all of the fractions. Absorbance was measured by flow through cell at 280nm.

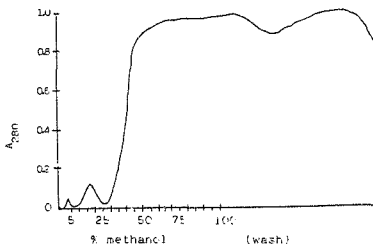


Figure 14. Elution profile of a medium gradient from an XAD-2 column: In an attempt to separate cAATrp from the other secondary metabolites of *P. cyclopius* Amberlite XAD-2 was used as the packing. The sample was added to the column (30 x 15cm) via an LKB varioperpex pump. The column was eluted with 20ml aliquots of methanol, in stepwise increases of 5%. α - and β -CA were found in all of the fractions. Absorbance was measured by flow through cell at 260nm.

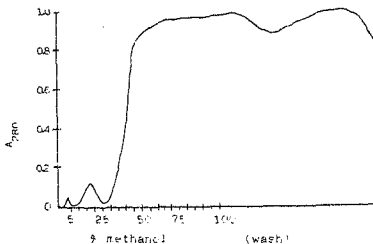


Figure 14. Elution profile of a medium gradient from an XAD-2 column: In an attempt to separate cAATp from the other secondary metabolites of *P. cyclopius* Amberlite XAD-2 was used as the packing. The sample was added to the column (30 x 15cm) via an LKB varisuperpex pump. The column was eluted with 20ml aliquots of methanol, in stepwise increases of 5%. α - and β -CA were found in all of the fractions. Absorbance was measured by flow through cell at 280nm.

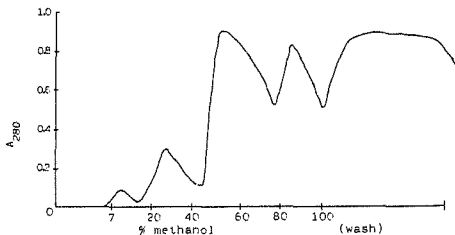


Figure 15. Elution profile from a steep gradient from an XAD-2 column: In an attempt to separate cAA_{trp} from the other secondary metabolites of *P.cyclopium* Amberlite XAD-2 was used as the packing. The sample was added to the column (30 x 15cm) via an LKB verierpex pump. The above column was eluted with a 50ml aliquot of 7% methanol followed by 50ml aliquots of each of 20, 40, 60, 80 and 100% methanol. Detection was by flow through cell at 280nm. α - and β -CA were found in all of the fractions.

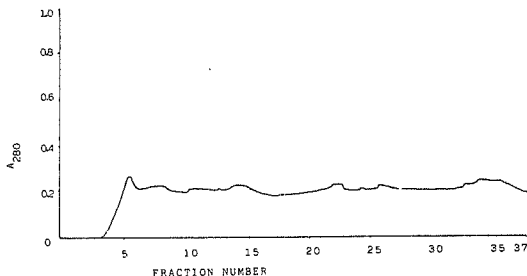


Figure 16. Elution profile from a silica gel column: In an attempt to separate cAAtrp from the other secondary metabolites of *P.cyclopium* Silica gel (Kieselgel) was used as the packing. The sample was added to the column (30 x 15cm) via an LKB varioperpex pump. The above column was developed using ethylacetate/ammonia/methanol (95:2.5:2.5) and the fractions read using a Beckman DB spectrophotometer. α - and β -CA were found in all of the fractions.

that Ca^{2+} is used in the t.l.c. plates to fix the silica to the glass plate, whilst it is not present in the column. Thus the separation seen on the t.l.c. plates may be due more to the chelation effects of α^- and β^- CA than the normal t.l.c separation effects. As seen with the XAD-2 the CA bound strongly to the silica gel and was not washed off with either methanol or propanol and as successive samples were added to the column so the contamination of the fractions by CA became larger. In later experiments using the silica gel column 2.5% ammonia (25%) was used in the elution buffer in an attempt to enhance the separation as the ethylacetate/methanol do not, when combined, make a satisfactory elution buffer (Fig 16).

A Sephadex G10 packing was then used, as it was thought that the aromatic binding properties of Sephadex G10 as well as its filtration effects might enhance separation (see below). 1mM EDTA was added as the CA is known to be a strong chelator of Cu^{2+} and Mg^{2+} (Steyn and Rabie 1976; Holzapfel 1980), and it was thought that the chelating effect of CA may have been the cause of its strong binding to all the column packings. Again it was found that irrespective of the concentration of methanol, formic acid or ammonia used to wash the column, the CA remained bound to the column.

It was then decided to use 50% acetone adjusted to pH 2 to attempt to release the CA from the column, and this was found to work as a large surge of CA was eluted from the column after its addition. When the full 300ml of 50% acetone pH2 had been passed through the column, various other buffers were run through the column, and no elution of CA could be detected as had previously occurred.

When using the low water regain Sephadexes (G10, G15 or G25) care must be taken as effects other than molecular sieving have been observed. The molecular sieving properties of the dextran gels received attention first, later however various other interactions were observed. Porath (1960) and Gelotte (1960) described a number of observations where substances showed a behaviour differing widely from that which might be ex-

pected from their molecular size. That is their behaviour could not be interpreted as only a restricted diffusion into and through the gel phase caused by steric hindrance.

Williams (1972) checked the solute-gel interactions in gel filtration in various types of gels. He looked at the dextran (Sephadex), polyacrylamide (Bio-Gel), and agarose (Sephacrose & Bio-Gel) gels.

The interactions between solutes and gels may be of a reversible or irreversible nature, the latter being uncommon and not well categorised. Some authors have reported losses of small amounts of protein on freshly packed Sephadex columns (Stevenson 1968). George (1962) reported that ions of heavy metals adsorb irreversibly to Sephadex. Similar adsorption has been found with dyes and some aromatic species. The reversible interactions may be classified into three main categories.

1. Ionic Interactions

The Sephadex and Bio-Gel P gels contain a low number of carboxylic groups in the matrix. Thus, in distilled water or eluants of low ionic strength, they can exhibit cation exchange properties. As the carboxyl content is almost constant throughout the gel ranges, the charge density, in the swollen gel, is higher in the lower water regain gels, i.e. it is more noticeable in Sephadex G10 than it is in Sephadex G200. In solutions of low ionic strength, acidic species are excluded from the gel, and thus their elution is speeded. Conversely the elution of basic material is retarded.

Gelotte (1960) reported the exclusion of amino acids from Sephadex in low ionic strength eluants. Similarly the exclusion of inorganic anions from the gel is well known (Spitzzy *et al* 1961). The retention of basic proteins on Sephadex was utilized by Miranda *et al* (1962) to separate scorpion venom toxins. Bio-Gel also shows an affinity for basic materials (Schwartz *et al* 1965).

2. Aromatic Interactions

The interaction of aromatic and heterocyclic compounds is well known for both Sephadex (Gelotte 1960; Porath 1968; Determann and Walter 1968) and polyacrylamides (Sun and Sehon 1965; Schwartz and Zabin 1966; Determann and Walter 1968). Consideration of the nature of aromatic species and possible mechanisms of interaction suggest involvement of the π electron system of the solute and an electron deficient or electronegative portion of the gel. In the case of the Sephadex gels, interactions would occur with the ether oxygens in the glyceryl bridge. Hydrogen bonding between substituents of aromatic compounds or the hetero-atom of heterocyclics and the glyceryl bridge in Sephadex may also occur. Eaker and Porath (1967) suggested the involvement of the π electron system of aromatic solutes in interaction effects. Determann and Walter (1968) provided evidence that the site of interaction in Sephadex was the glyceryl bridge. Brooks and Housley (1969) suggested that phenols could interact with Sephadex by hydrogen bonding between the hydroxyl group of the phenol and the glyceryl bridge of the matrix. Sweetman and Nyhan (1971) using Sephadex G10 found evidence that purines interact with the dextran chain. It is suggested that the interaction is between the hydroxyl groups of the Sephadex dextran chain and the ring nitrogen. Involvement of the π system is also thought to occur. Additional forces, dipole-dipole, dipole-induced dipole and dispersion forces might be affecting the interaction.

3. Specific Interactions

Specific interactions between solute and gels have been reported, for example, adsorption of pancreatic amylase to Sephadex G100 (Wilding 1963) and G75 (Gelotte 1964) is thought to be due to the formation of a mock enzyme/substrate complex. Concanavalin A has an affinity for D-mannoside and D-glucoside groupings in Sephadex G50 and Concanavalin A is purified by its adsorption to Sephadex G50, elution

being effected with glucose. The adsorption of aliphatic alcohols (Marsden 1965) Borate and hydroxyl ions to Sephadex is thought to be due to reactions with the hydroxyl groups of the dextran chain.

Thin Layer Chromatography (t.l.c.) plates of the eluted metabolites.

The thin layer chromatograms for the "Amberlite" XAD-2 columns, the silica gel columns and some of the Sephadex G10 columns are not included as they showed only α - and β -CA and traces of intermediates. All of the fractions from the XAD-2 columns were contaminated with α - and β -CA which was being bound over after each run, even after washing with 100% methanol and propanol. The results shown here are from newly packed columns, as it was found that once a column (XAD-2) had been used, bound α - and β -CA could not be removed without destroying the resin.

It is interesting to note that at various concentrations of methanol peaks of α - and β -CA washed off, and when run on t.l.c. plates showed slightly different R_f 's. More accurate work would have to be done on these structures though to see if they are various isomers of α - and β -CA.

The silica gel column results are included but it must be remembered that the column elution was not easily reproducible due to the effects of ethylacetate on the monitor. Fractions (5ml) were measured in a Beckman D.B. spectrophotometer, the samples showing absorbance were run on t.l.c. plates and again contamination of all absorbing fractions by α - and β -CA was seen.

With Sephadex G10 packing the first two experiments again showed contamination of all fractions with α - and β -CA, α -CA in particular. As can be seen from the results presented in Figures 17 and 18. However with the third experiment (Figure 19) although contamination of the fractions by α - and β -CA occurred, some separation of the intermediates could be seen (t.l.c. Figure 19). In Figures 20 and 21 formic acid was used with the ammonia, and it was seen that separation was not enhanced but was re-

tarded, and contamination by α - and β -CA again became noticeable (t.l.c. Figures 20 and 21).

Thereafter the column was run using 250mM ammonia with the EDTA (Figure 22 and t.l.c. Figure 22). This gave results similar to those seen in Figure 19 showing separation of the metabolites. This separation was improved by using the "standard" 1mM EDTA adjusted to pH 10.5 with ammonia (Figure 23 and t.l.c. Figure 23). Using this system cAATrp, trp-y and trp-z could be isolated with ease and accuracy.

Two t.l.c. plates were developed using the fractions marked on Figures 22 & 23. One was stained with Ehrlich's reagent and the other with ferric chloride (Silber and Shulze 1953) with which cAATrp stains as a very dark band. It is interesting to note that in those fractions containing intermediates many more bands are seen than intermediates so far suggested. It would be interesting to isolate these bands in quantities large enough to be assayed, as with the above technique will now be possible.

It was eventually found that the best buffer for effecting the elution of cAATrp was 1mM EDTA adjusted to pH 10.5 with ammonia. It can be seen that trp y and z were eluted first followed by cAATrp, immediately after cAATrp was eluted the column was washed with 50% acetone pH 2 to remove all of the α - and β -CA. The use of 50% acetone pH 2 to remove the α - and β -CA from the column show that the separation was not brought about by molecular sieving alone, but more probably by separation according to the charge carried by the molecules in the sample.

This means that it is now possible to obtain large amounts of cAATrp one of the substrates for the secondary transferase, also large amounts of the unknown intermediates can be purified using preparative t.l.c. and thus their structures can be elucidated. It would appear from the literature that the separation observed is probably due to the aromatic interactions that occur in the gel.

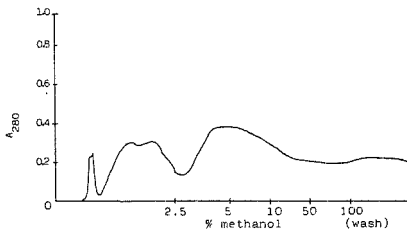


Figure 17. Elution profile of a shallow methanol gradient from a G10 column: The sample was added to the column (30 x 15cm) via an LKB verierpex pump. The above column was eluted with 50ml aliquots of each of 2.5%, 5%, 10% and 50% methanol, the column being washed with 100ml of 100% methanol followed by 100ml of distilled water. Detection was by flow through cell at 280nm. α - and β -CA were found in all of the fractions

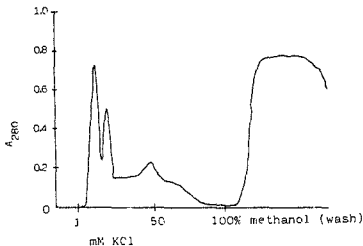


Figure 18. Elution profile of a shallow KCl gradient on a G10 column: The sample was added to the column (30 x 15cm) via an LKB varioperpex pump. The above column was developed with 300ml of 1mM EDTA, 1mM KCl followed by 100ml of 1mM EDTA, 50mM KCl. At the end of the run the column was washed with 100ml of 100% methanol followed by 100ml of distilled water. Detection was by flow through cell at 280nm. α - and β -CA were found in all of the fractions.

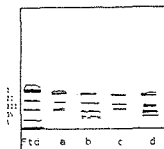
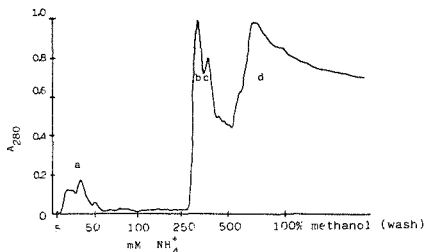


Figure 19. Elution profile of a steep ammonia gradient from a G10 column: The sample was added to the column (30 x 15cm) via an LKB varioprep pump. The above column was developed with 50ml of 5mM, 50mM, 100mM 250mM and 500mM ammonia. At the end of the run the column was washed with 100ml of 100% methanol followed by 100ml of distilled water. Detection was by flow through cell at 280nm. In the thin layer chromatogram, the standard is the sample before addition to the column, I is α - and β -CA, II is cAAtrp, III is trp-y, IV is trp-z, V is tryptophan.

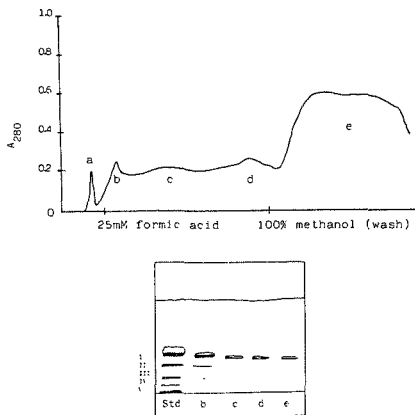


Figure 20. Elution profile of a shallow formic acid gradient from a G10 column: The sample was added to the column (30 x 15cm) via an LKE varioperex pump. The above column was eluted with 300ml of 1mM EDTA, 25mM formic acid adjusted to pH 10 with ammonis. At the end of the run the column was washed with 100ml of 100% methanol followed by 100ml of distilled water. Detection was by flow through cell at 280nm. In the thin layer chromatogram, the standard is the sample before addition to the column, I is α - and β -CA, II is cAAtrp, III is trp-y, IV is trp-z, V is tryptophan.

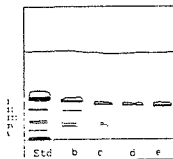
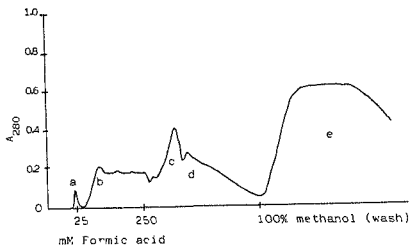


Figure 21. Elution profile of a steep formic acid gradient from a G10 column: The sample was added to the column (30 x 15cm) via an LKB varioperpex pump. The above column was eluted with 200ml of 1mM EDTA, 25mM formic acid adjusted to pH 10 with ammonia, followed by 100ml of 1mM EDTA, 250mM formic acid adjusted to pH 10 with ammonia. At the end of the run the column was washed with 100ml of 100% methanol followed by 100ml of distilled water. Detection was by flow through cell at 280nm. In the thin layer chromatogram, the standard is the sample before addition to the column, I is α - and β -CA, II is cAAtrp, III is trp-y, IV is trp-z, V is tryptophan.

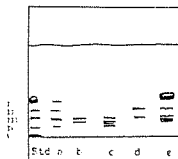
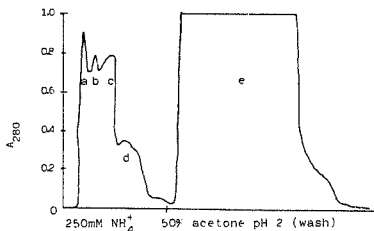


Figure 22. Elution profile of a medium ammonia gradient from a G10 column: The sample was added to the column (30 x 15cm) via an LKB varioperpex pump. The above column was eluted with 300ml of 1mM EDTA, 250mM ammonia, and washed with 50% acetone adjusted to pH 2 using HCl. Detection was by flow through cell at 280nm. In the thin layer chromatogram, the standard is the sample before addition to the column, I is α - and β -CA, II is cAAtrp, III is trp-y, IV is trp-a, V is tryptophan.

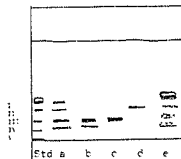
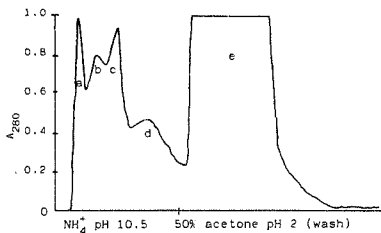


Figure 23. Elution profile of an ammonia adjusted EDTA eluant from a G10 column: The sample was added to the column (30 x 15cm) via an LKB varioprep pump. The above column was eluted with 300ml of 1mM EDTA adjusted to pH 10 - 10.5 using ammonia. At the end of the run the column was washed with 100ml of 50% acetone adjusted to pH 2 with HCl followed by 100ml of distilled water. Detection was by flow through cell at 280nm. In the thin layer chromatogram, the standard is the sample before addition to the column, I is α - and β -CA, II is cAATrp, III is trp-y, IV is trp-z, V is tryptophan.

It can be seen from the t.l.c. plates that the bands when run, appeared in pairs or triplicates in the immediate vicinity of their positions on the thin layer chromatogram. It is possible that these bands may be isomers of each other, see Figure 12.

3.7 Inhibitor Studies on *Penicillium cyclopium* using Clofibrate

Clofibric acid (2-(p-chlorophenoxy)-2 methylpropionic acid) was used in an attempt to increase the pool of cAAtp by inhibiting HMG-Co-A reductase (Rodwell, M^c Namara and Shapiro 1973). If the HMG-Co-A reductase is blocked no IPP production can occur and thus no DMAPP production. This would lead to an increase in the amount of AcCoA present, and therefore possibly an increase in cAAtp as no DMAPP would be present to produce β -CA. It has been shown that Clofibric acid and its ethyl ester (CPIB) lower plasma cholesterol levels (Nestle *et al* 1965) and are in clinical use. Burch and Curran (1969) suggested that Clofibrate blocks cholesterol synthesis by increasing the rate of deacylation of acetoacetyl-CoA (See Figure 24). White (1971) has shown that the major effect of Clofibrate is at HMG-Co-A reductase and that a 0.3% Clofibrate diet produces a 60 - 90% decrease in reductase activity within 3 days.

Various concentrations of Clofibrate (46mM; 4.6mM; 0.46mM; 0.046mM; 0.00mM) were applied to *P.cyclopium* over five days and the growths checked for α - and β -CA production, as well as for cAAtp to check for the predicted increase in pool size.

The cultures were grown and sub inoculated, as described (2.6), 24 hours later the first Clofibrate was added and thereafter samples were taken every 24 hours and more Clofibrate, at its respective concentration, added. See Table 8 and Figures 25 and 26 for results.

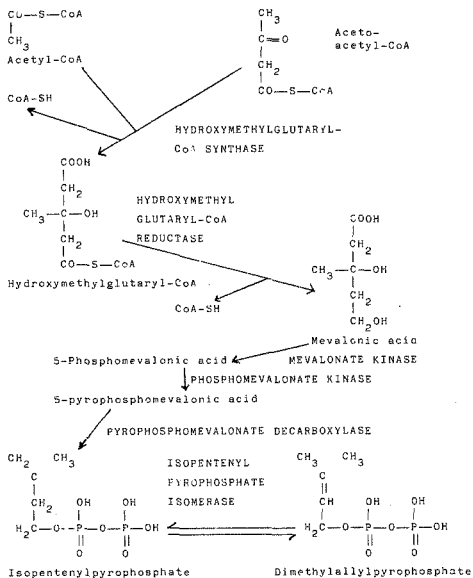


Figure 24. Route of metabolism to steroid synthesis. The involvement of HMG-CoA reductase in steroid biosynthesis.

Thin layer chromatograms of the products derived from inhibitor studies

In order to check that it was CA being produced and not a build up in cAAtRp (the desired effect) causing the increased values. The log molar extinction coefficients for CA and cAAtRp are similar. t.l.c. plates were run on the extracts of the organic layer after the mycelium had been homogenised and the extract centrifuged (see Materials and Methods 2.6). Figure 27 shows the thin layer chromatogram of the metabolites extracted from *P.cyclopium* treated with Clofibrate.

The chromatograms of extracts derived from the inhibition studies at:

Day 2; shows the organic extract running from the blank on the left to the highest concentration of Clofibrate on the right. It can be seen that all the intermediates and α - and β -CA are present.

Day 3; It can be seen in the control that the appearance of α - and β -CA and the intermediates are normal, whereas a large decrease in their concentrations can be seen in the Clofibrate treated *P.cyclopium*.

Day 4; The Clofibrate treated growths and the blank showed approximately the same amounts of α - and β -CA, but the intermediates from the Clofibrate treated growths showed signs of decreasing. The 0.46mM treatment did however show a larger amount of cAAtRp than the other Clofibrate treatments, almost half the size of the band for the blank (visual check only).

Day 5; It can be seen now that the α - and β -CA concentration in the Clofibrate treated growths has reached that found in the blank, and has in fact exceeded production by the blank in all treatments (see Table 8). However the intermediates of CA production decreased drastically in the Clofibrate treated growths, and had in some cases disappeared, although it was noticed that many more bands were seen albeit that they were very faint.

In Figures 25 and 26 it can be seen that the concentration of CA had not altered significantly in any of the treatments by day two. However it can be seen that by day three the Clofibrate seemed to be having an effect, as the concentration of all the secondary metabolites had decreased compared to the blank (and Figure 27, day 3, confirms this). By day four the concentration of the metabolites had returned to that of the control, and by day five had surpassed that of the control, this might suggest that cAATrp had indeed pooled, yet it was only the concentration of α - and β -CA that had increased appreciably (see t.l.c. 4 and 5, Figure 27). Throughout these changes the dry weight of the mycelial mats did not differ significantly from that of the control. Taking the results of day three it would appear that Clofibrate does have an inhibitory effect on the HMG CoA reductase in *P. cyclopium*, and that for some reason this control is bypassed. It may be possible that there is a pool of DMAPP or IPP present in the organism to begin with, and this becomes available to the enzymes as the organism progresses deeper into the idiophase, where it is known that physiological changes occur (Bu'Lock 1965, 1975; Woodruff 1966; Berry 1975). It is however highly unlikely that the DMAPP will pool as Mc Grath *et al* (1976) showed that DMAPP was the rate limiting metabolite. An alternative hypothesis is that during the change to the idiophase and the production of secondary metabolites, it is possible that previously unavailable enzymes and substrates may become available. Thus an alternative route may be created for the production of IPP or DMAPP or some of the other metabolites involved in the production of CA. Another possibility is that there is input from fatty acid breakdown at some point after the clofibrate has had its action.

It is interesting to note from the t.l.c. plates, day four and five (Figure 27), that the only secondary metabolite in the Clofibrate treated growths to show an increase is in fact α - and β -CA (which is why this method could not be used to increase the amount cAATrp, to facilitate its extraction). Thus independently of where the substrates for α - and β -CA are from, some factor brought on by the presence of Clofibrate is causing an increase in the catalytic activity of the secondary transferase.

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TABLE 5 Clofibrate Inhibitor Studies on *P. cyclopius*

Time hours	Concentration Clofibrate applied	total CA in μ g	dry weight mycelial mat in gm
48 hours	46mM	805	0.945
	4.6mM	488	0.82
	0.46mM	667	0.9
	0.046mM	666.8	0.92
	0.000mM	671	1.075
72 hours	46mM	179	1.205
	4.6mM	354	1.185
	0.46mM	318	1.145
	0.046mM	420	1.125
	0.000mM	1092	1.275
96 hours	46mM	850	1.345
	4.6mM	1092	1.345
	0.46mM	1194	1.35
	0.046mM	1218	1.43
	0.000mM	1110	1.42
120 hours	46mM	2548	1.32
	4.6mM	1987	1.4
	0.46mM	2187	1.31
	0.046mM	2020	1.335
	0.000mM	1832	1.36

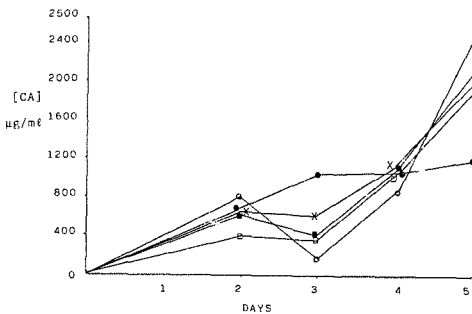


Figure 25. Clofibrate Inhibitor Studies: The above shows the concentration of both α and β CA in $\mu\text{g/ml}$ over 120 hours for all samples

- 0.00mM Clofibrac acid (blank)
- 0.046mM Clofibrac acid
- X 0.46mM Clofibrac acid
- 4.6mM Clofibrac acid
- 46mM Clofibrac acid

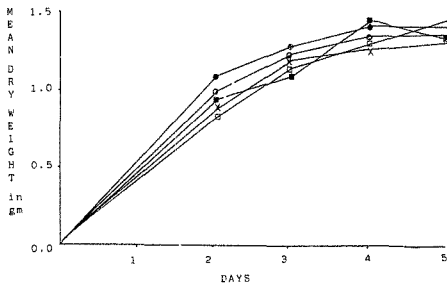
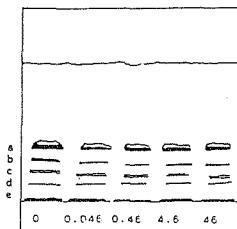
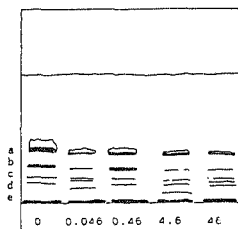


Figure 26. Clofibrate Inhibitor Studies: Mycelial mat dry weight (gm) over the period of the experiment for all samples, showing no significant difference in mean dry weights.

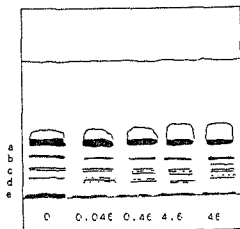
- 0.00mM Clofibric acid
- 0.046mM Clofibric acid
- X 0.46mM Clofibric acid
- 4.6mM Clofibric acid
- 46mM Clofibric acid



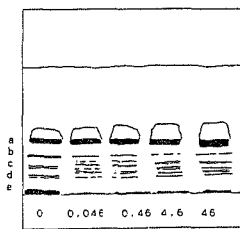
DAY 2



DAY 3



DAY 4



DAY 5

Figure 27. Thin layer chromatograms of the metabolites extracted from *P.cyclopium* treated with Clofibrate. : The figures at the bottom of the individual streaks on each t.l.c. plate denote the mM Clofibrate concentration used. In the thin layer chromatogram a is α - and β -CA, b is cAATrp, c is trp-y, d is trp-z and e is tryptophan.

CHAPTER 4

CONCLUSIONS

CONCLUSIONS

1. It was found that the best method of cell rupture was the acetone/dry powder method. Enzymes isolated in this manner retained their activity for at least a year when stored at -20°C . This method also had the advantage that contaminating lipids and various other metabolites would be removed in the acetone. The dried powder is easily stored and reconstitution of the powder proved to be easy, and once reconstituted the enzymes were stable.
2. The secondary transferase is best produced using nutrient rich corn steep liquor. This result suggests that, in this case, the production of cyclopiazonic acid is not controlled by enzyme concentration (this supports the results of M^c Grath *et al* 1976 and 1977). This is so because the secondary transferase is present in high concentrations in mycelium grown on rich medium even though the secondary metabolite production is repressed. The explanation may well be that under nutrient rich conditions cAATrp, a precursor of the secondary transferase, is absent. It is also possible that under these conditions DMAPP, the other precursor of the secondary transferase, remains, to a large extent, involved in primary metabolism and the production of farnesylpyrophosphate, rather than being siphoned off into secondary metabolism.
3. cAATrp is produced and purified in mycelium grown on well defined media using NaNO_3 as the nitrogen source. The isolation of cAATrp is best performed using Sephadex G10 and eluting with 1mM EDTA adjusted to pH 10-10.5 with ammonia. The forces involved in this separation are not fully understood, but may be due to the aromatic interactions which are known to occur in Sephadex G10.

4. It was believed that inhibition of the enzyme HMG-CoA reductase using Clofibrate would lead to an increase in cAAtrp, by both forcing AcCoA into this pathway and by preventing the appearance of DMAPP. However this did not occur, possibly because Clofibrate has no effect in *Penicillium cyclopium*. It should be noted however that, even though less cAAtrp is present, the level of both α - and β -CA is higher by day 5 in the Clofibrate treated cultures than in the controls. It is possible perhaps that the secondary transferase is reacting faster under these conditions and thus more α - and β -CA is being produced and the cAAtrp is being utilised as soon as it becomes available i.e. DMAPP is being produced in larger amounts.
5. The isomerases and primary transferases isolated from both pig liver and *Penicillium cyclopium* are very alike. They show the same isoelectric points, similar molecular weights and the enzymes are interconvertible, the enzymes from one being used in enzyme assays for the other.
6. In further research it would be very interesting to compare the prenyl-aryl transferase (S) with the prenyl transferase (T). It may well be that they have similar structures, and more work should be done on the genetics of *Penicillium cyclopium*.

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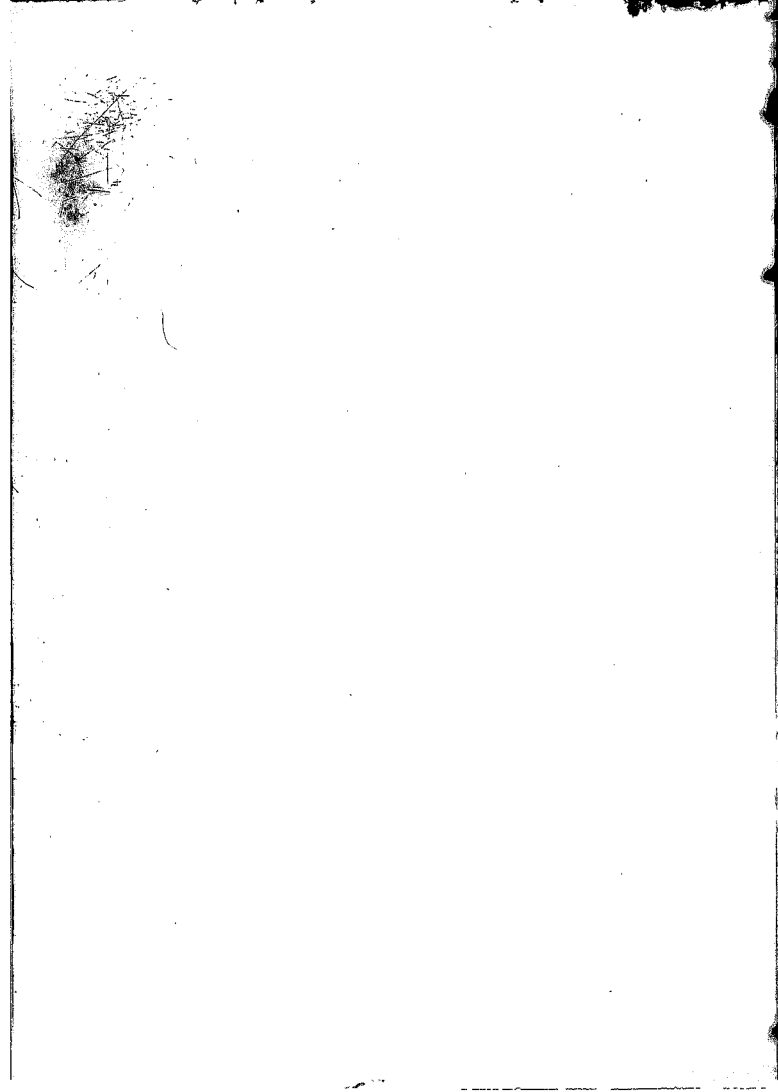
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